GREAT LAKES BIOENERGY RESEARCH CENTER

Developing genome-enabled sustainable lignocellulosic biofuels technologies

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PNNL Frontiers in Biological Sciences

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U.S. DEPARTMENT OF ENERGY

DOE Bioenergy Research Centers

THE UNIVERSITY of WISCONSIN MADISON

Great Lakes Bioenergy Research Center

- **1 of 3 Bioenergy Research Centers (BRC) established in Fall, 2007 (anticipate ~142M in support by 2012)**

- **Academic**
  UW-Madison (lead)
  Michigan State University
  Illinois State University
  Iowa State University

- **DOE Office of Science**
  Joint Genome Institute
  BACTER Institute
  ASCR

- **DOE National Labs**
  Pacific Northwest NL
  Oak Ridge NL

- **Wisconsin & Michigan**
  Facilities, Faculty & Staff

- **Industry**
  Lucigen/C5-6 Technologies

- **Tech Transfer**
  WARF, others

Operational Advantages

- Integrates “hand-picked” scientists (“>350”) across sites & cultures
- Leverages diverse approaches to achieve a shared strategic vision
  - Individual investigator creativity
  - Biological, physical & computational sciences
  - Wet, dry & field laboratories
  - High throughput core facilities
- Partners embrace mission, strategy & collaborative philosophy
- “Scientific canteens” empower teamwork & creativity
  - Collaborations invest in our strengths while tapping expertise of other centers (BESC, JBEI) & DOE labs (JGI, etc.)

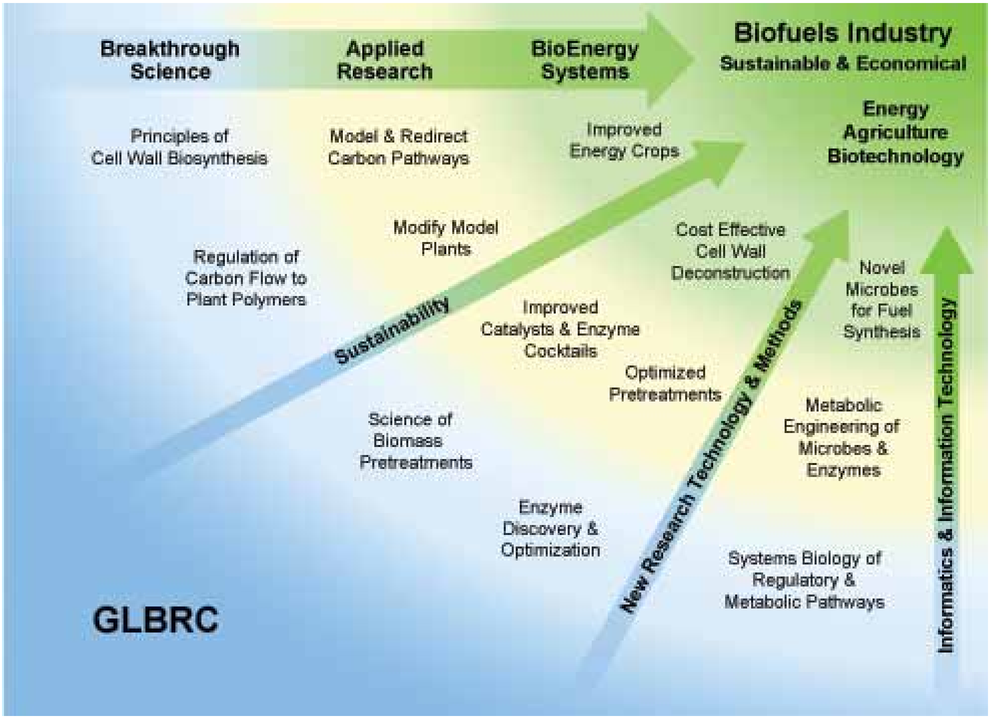
jbei Joint BioEnergy Institute

JGI DOE JOINT GENOME INSTITUTE US DEPARTMENT OF ENERGY OFFICE OF SCIENCE

BESC BioEnergy Science Center

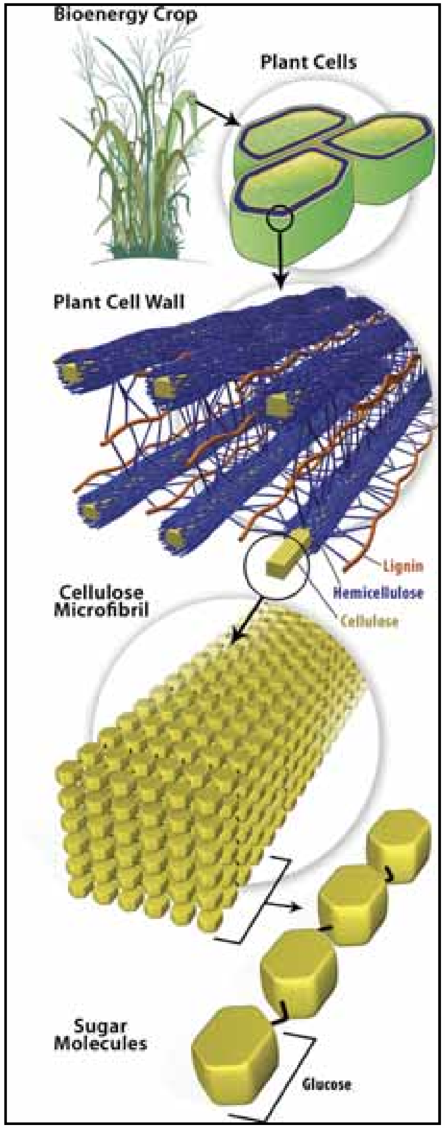
Great Lakes Bioenergy Research Mission

- **BRC Mission** "…produce fundamental scientific discoveries and major technological advances to **enable the development of cost-effective, energy-efficient, and commercially viable processes for large-scale conversion of lignocellulosic biomass into fuels**." [BRC Management Plan]



*"The **G**reat **L**akes **B**ioenergy **R**esearch **C**enter is a global leader in fundamental and applied research to catalyze a technically advanced biofuels industry that is economically & environmentally sustainable." [GLBRC Roadmap, November, 2008]*

Cellulosic Biofuels “Opportunities & Challenges”



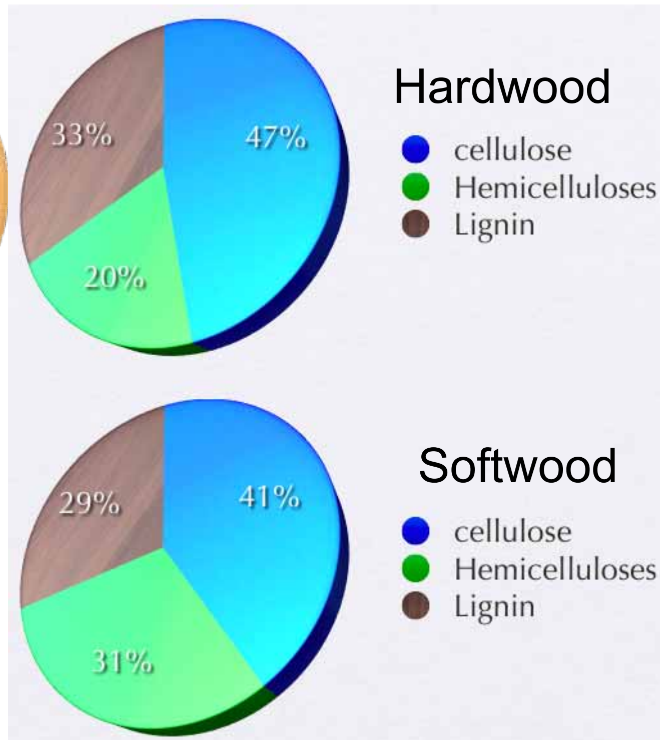
Opportunities:

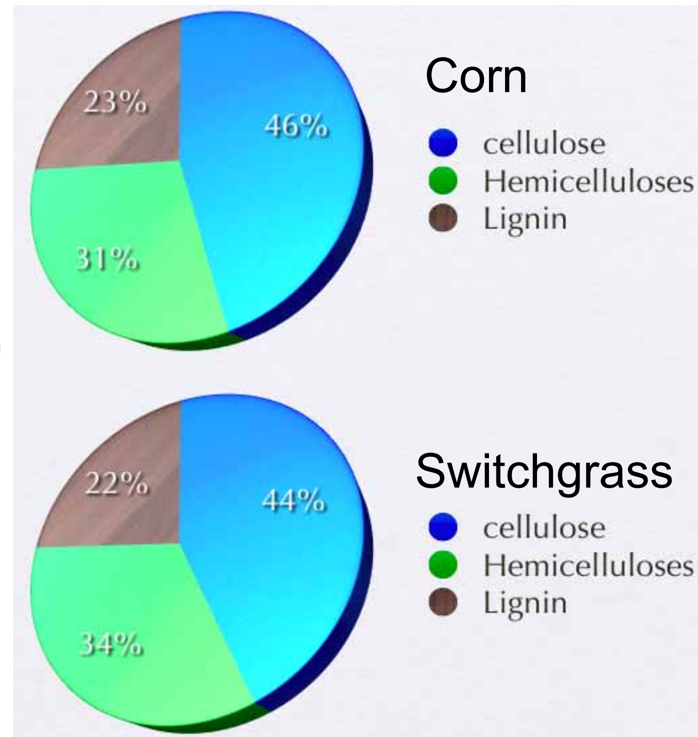
- **Non-edible** part of plants (negate “food vs. fuel”)
- **Most abundant** organic material on Earth (lots of it)
- Largely **sugar polymers** (glucose plus others) that can be converted to other fuels by catalytic or microbial chemistries

Challenges:

- Sugars trapped within insoluble fibers
- Fibers held together by hemicellulose (“difficult” sugars) & lignin (phenolic polymer)
- Not all plant cellulose is the same (different hemicellulose sugars, lignin content, etc.)

Variable Composition of Lignocellulosic Biomass





- Drives need for common & designer solutions

Today's Liquid Bioethanol Technology

Conversion of sugar cane (glucose) or corn starch (glucose polymer) to ethanol

Corn (kernels)

Starch

Heat or enzymes

Glucose

Fermentation

Ethanol

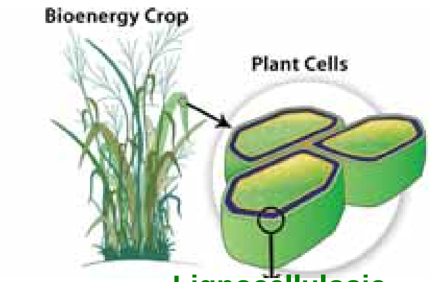
Sugar Cane

Glucose

Fermentation

Ethanol

Conversion of Cellulosic Plant Biomass to Fuels



Today's technology

Corn (kernels)

Starch

↓ *Heat or enzymes*

Glucose

↓ *Fermentation*

Ethanol

Sugar Cane

Glucose

↓ *Fermentation*

Ethanol

Tomorrow's technology (Great Lakes Bioenergy)

Cellulosic Biomass

↓ ? *Grind (reduce size)*

Pretreatment "soften cellulose"

↓ ? *Chemicals/heat*

Digest softened polymers

↓ ? *Enzymes*

Mixed sugar stream: Glucose, Arabinose, Xylose, Phenolics, etc.

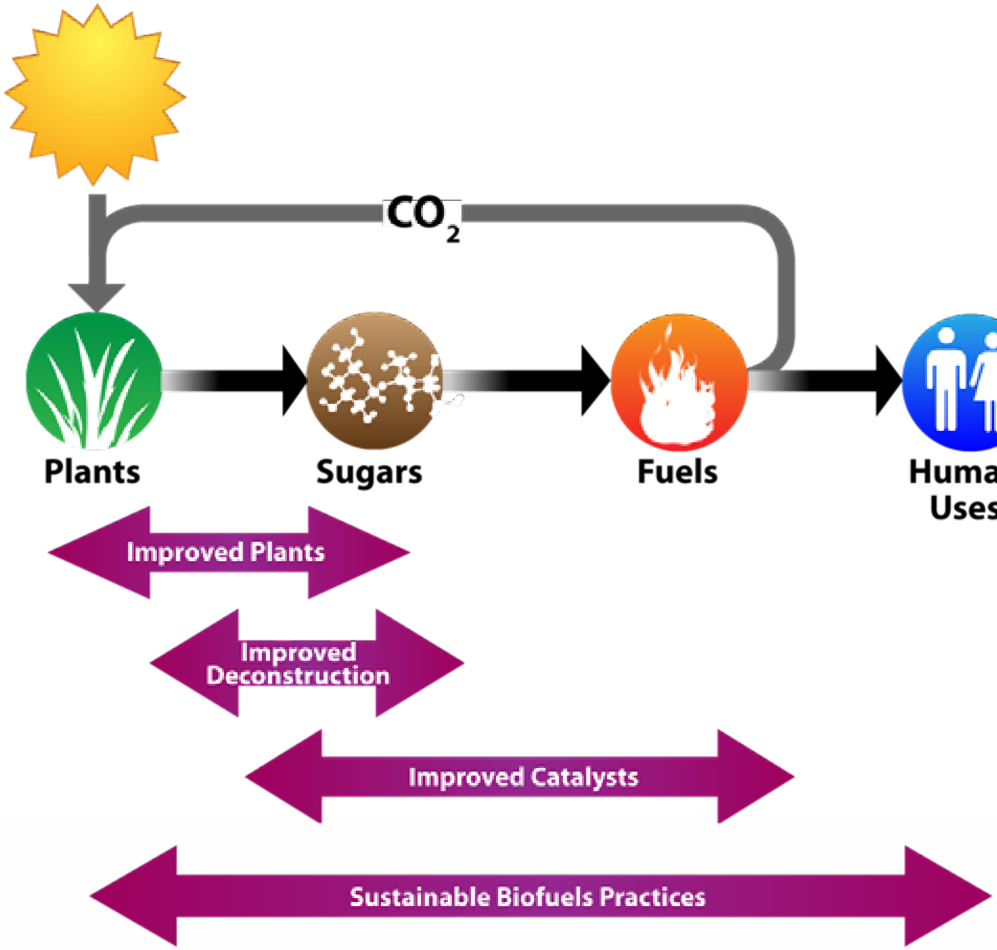
↓ ? *Fermentation/ Catalysts*

Ethanol, next generation fuels

Great Lakes Bioenergy Research Roadmap

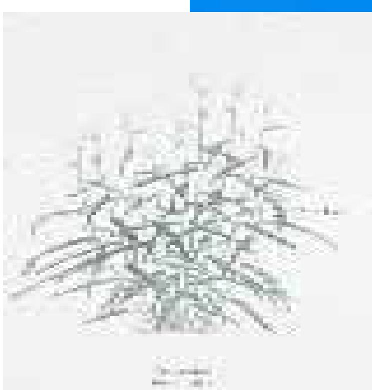
Fundamental science to sustainably convert cellulosic plant biomass into ethanol & next generation fuels

- Systems to achieve sustainable carbon & electron flow into fuels or co-products



Can We Improve Plants as Bioenergy Feedstocks?

- Biomass plants will need different traits than those traditionally used for food

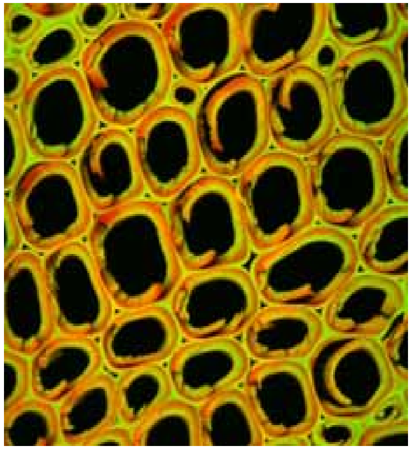


Teosinte → Maize

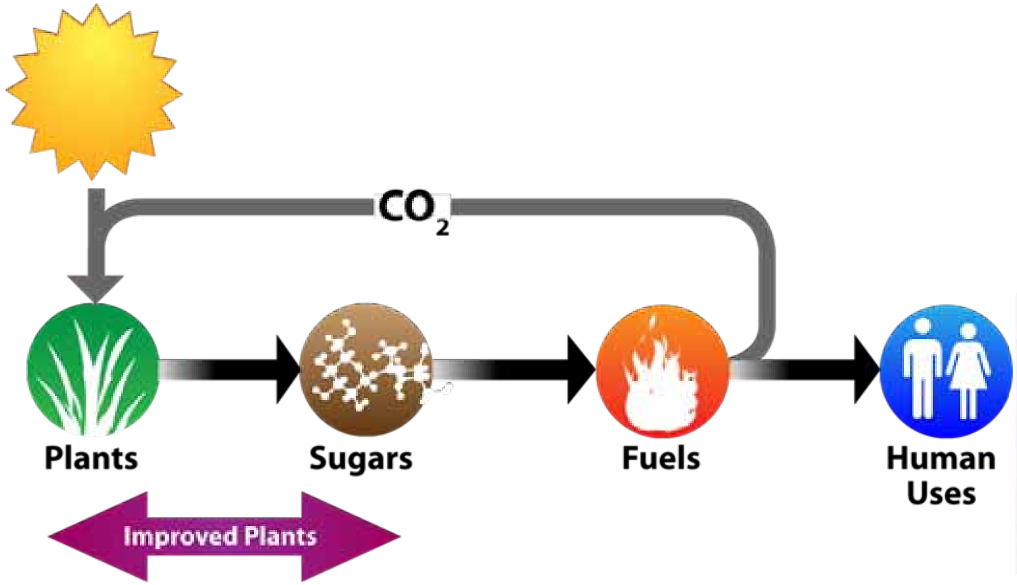
John Doebley, UW-Madison

- Only 5 genetic changes needed to develop modern Maize from Teosinte!!

Improving Energy Traits of Plant Biomass



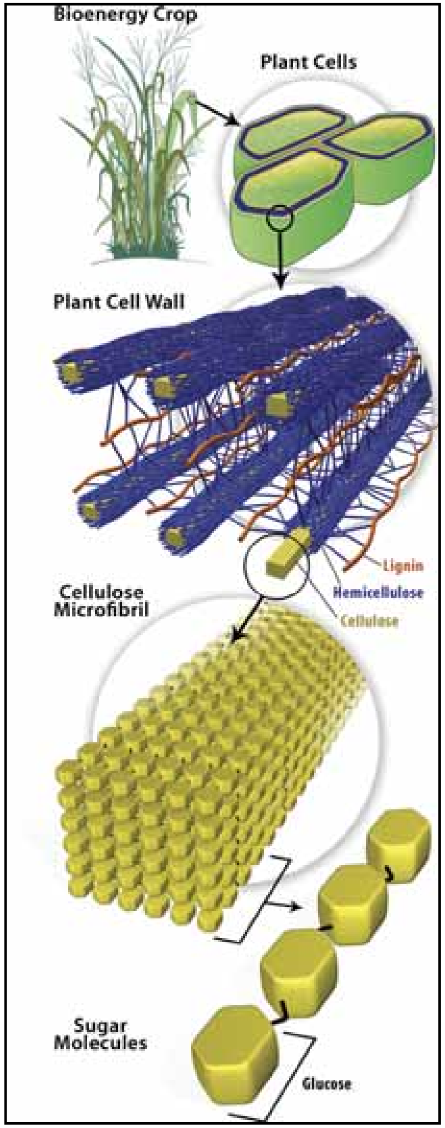
Plant Cell Wall



- Organization/composition of plant cell wall limits sugars for fuel production

Issue	Approach
Lignin blocks access to sugars	Lignin “Zips”
Hard to generate fuel from cell wall sugars	Modify hemicelluloses
Do not store other energy polymers	Accumulate oils, starch

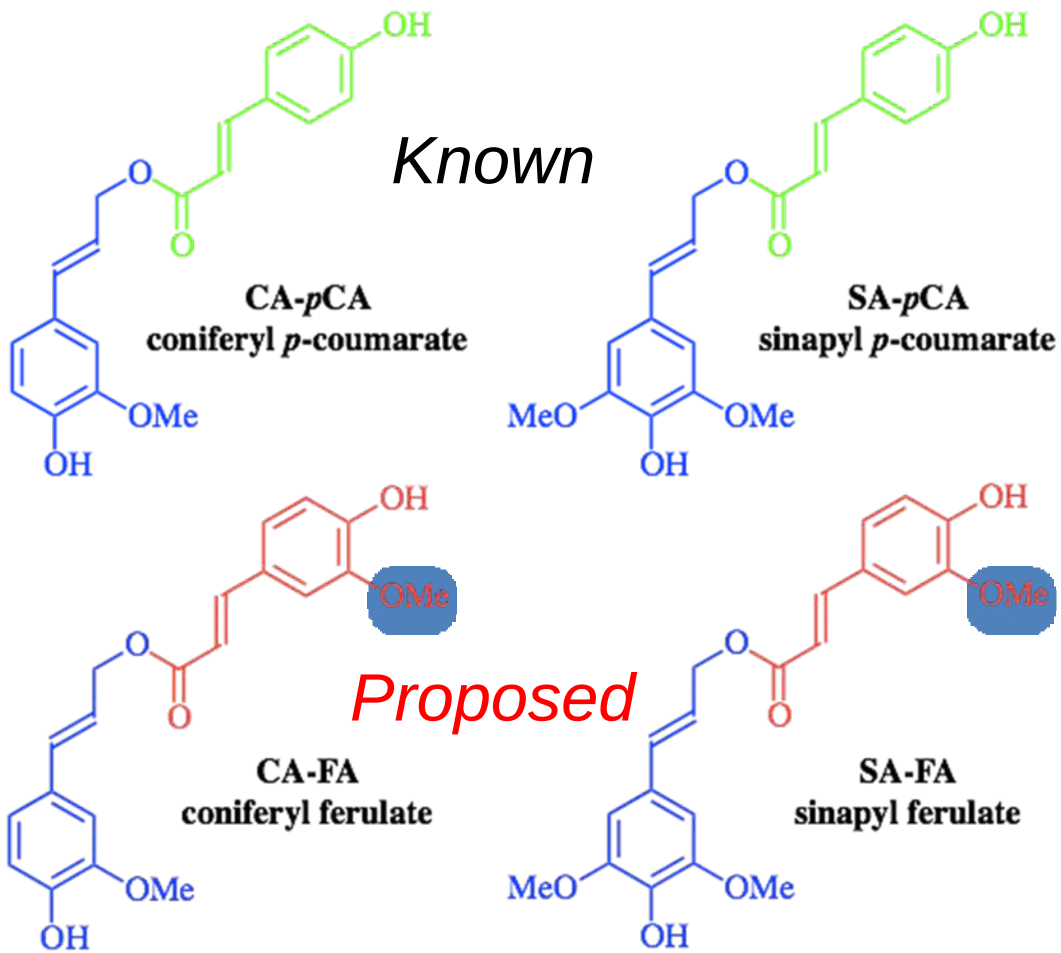
Redesigning Lignin for Improved Deconstruction



- Lignin blocks access to cell wall polysaccharides
- Removing lignin requires energetically expensive & chemically harsh pretreatments

- **Biomass will be better suited as a liquid fuel feedstock if the basic structure of lignin is altered** or the fraction of lignin is reduced
- Develop lignin that is optimized for both structural integrity & energy conversion

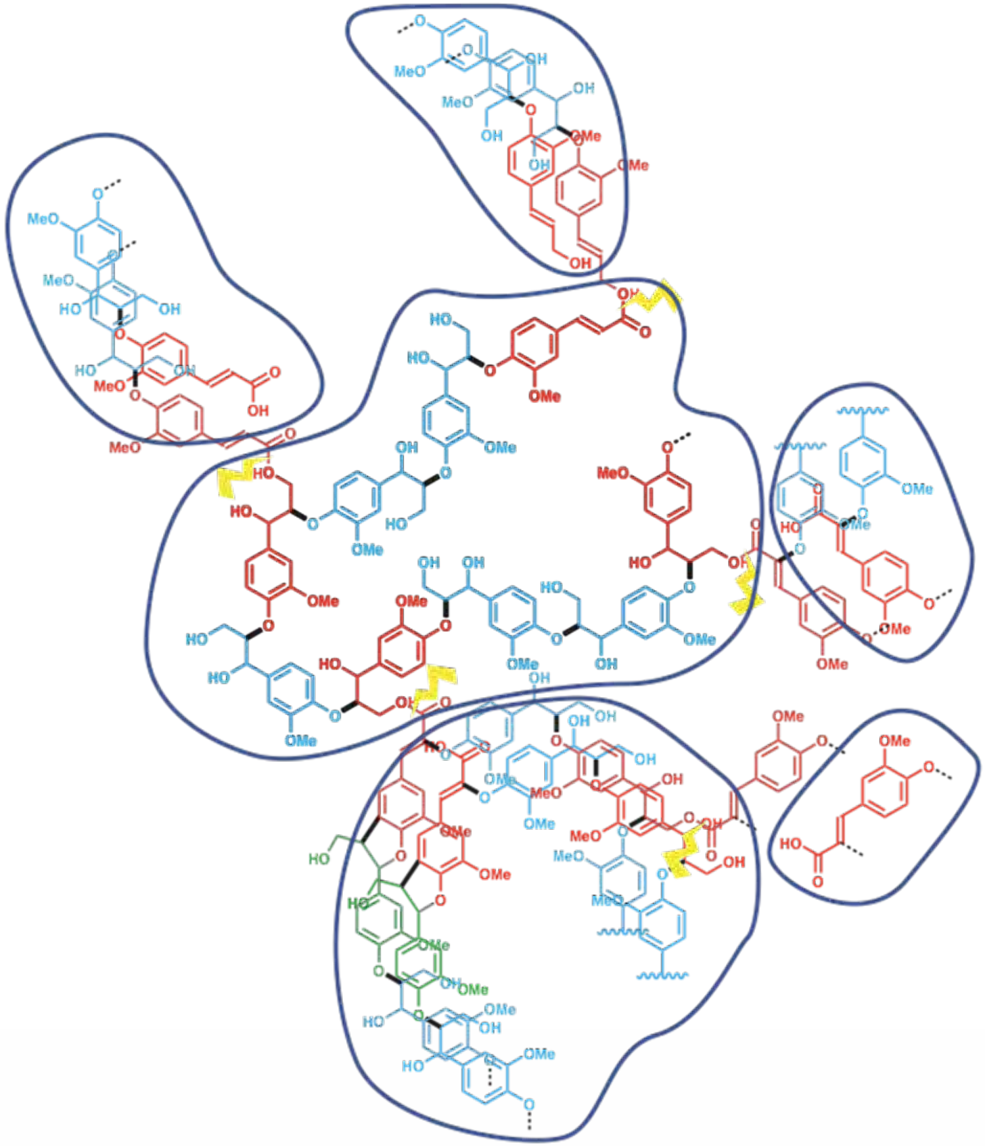
Alter Lignin Monomers to Create "Zippers"



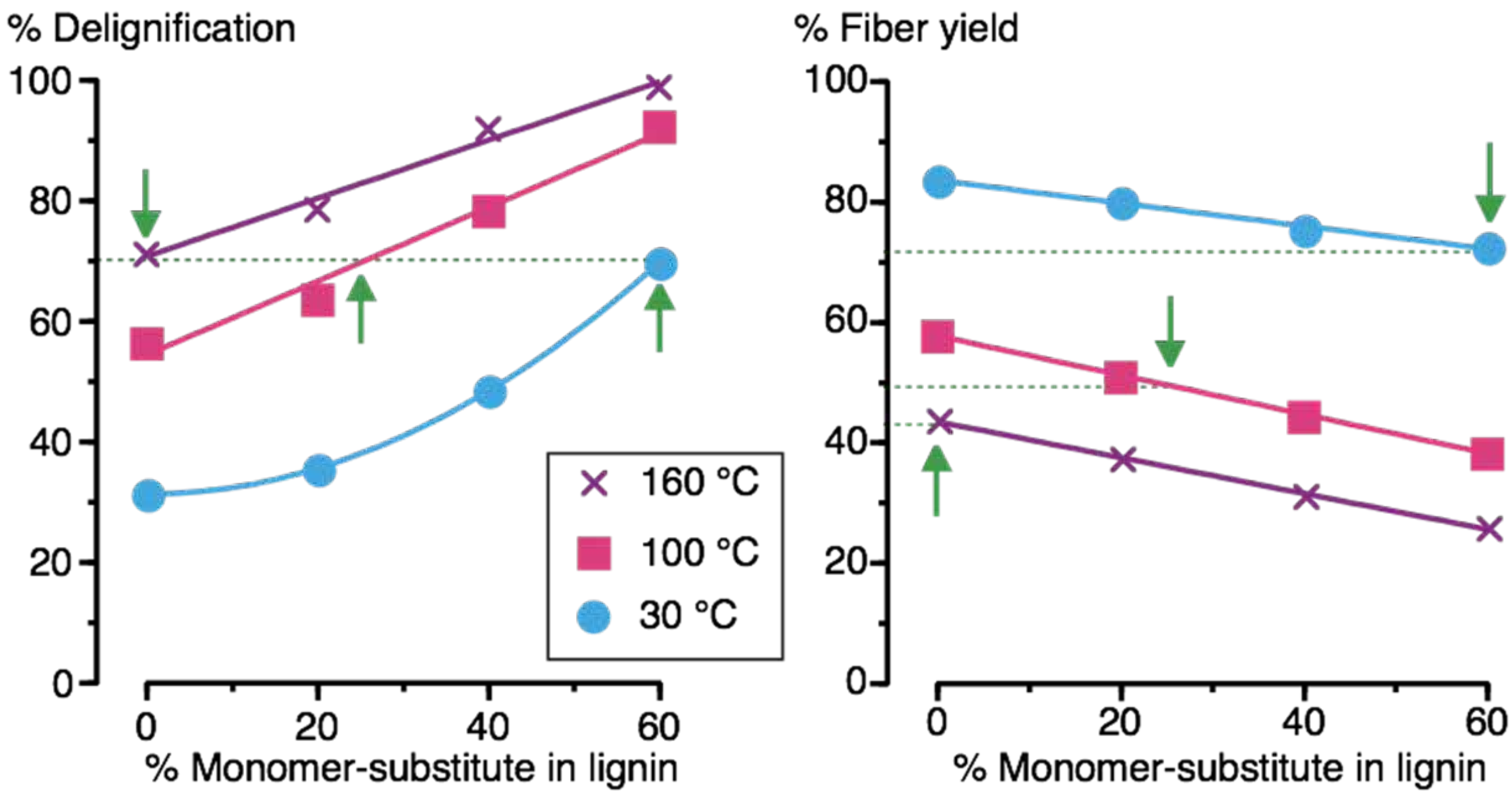
- Peroxidase adds exogenous ferulate monomers to lignin
- Replaces an ether linkage with an ester in polymer backbone

Grabber, J.H., Hatfield, R.D., Lu, F., Ralph, J. *Biomacromolecules* 9:2510-2516 (2008).

Ferulate esters provide cleavable lignin "zippers"



"Lignin Zippers" Reduce Energy Needed to Release Sugars

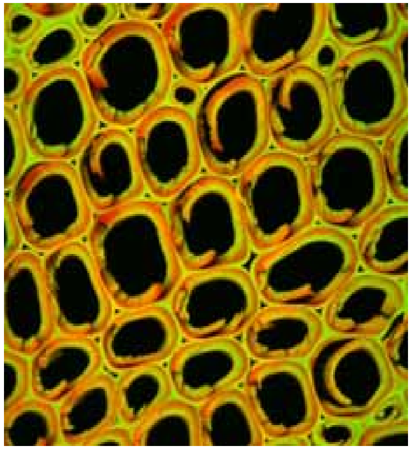


- Delignification at 100°C (or 30°C) as effective as at 170°C with natural lignins!
- Sugar yields from altered cell walls are higher than wild type at all temperatures!
- *Can we 'redesign lignin' for efficient conversion, reduced impact, local processing*

Grabber, J.H., Hatfield, R.D., Lu, F., Ralph, J. *Biomacromolecules* 9(9):2510-2516 (2008)**

**patents applied for

Improving Energy Traits of Plant Biomass



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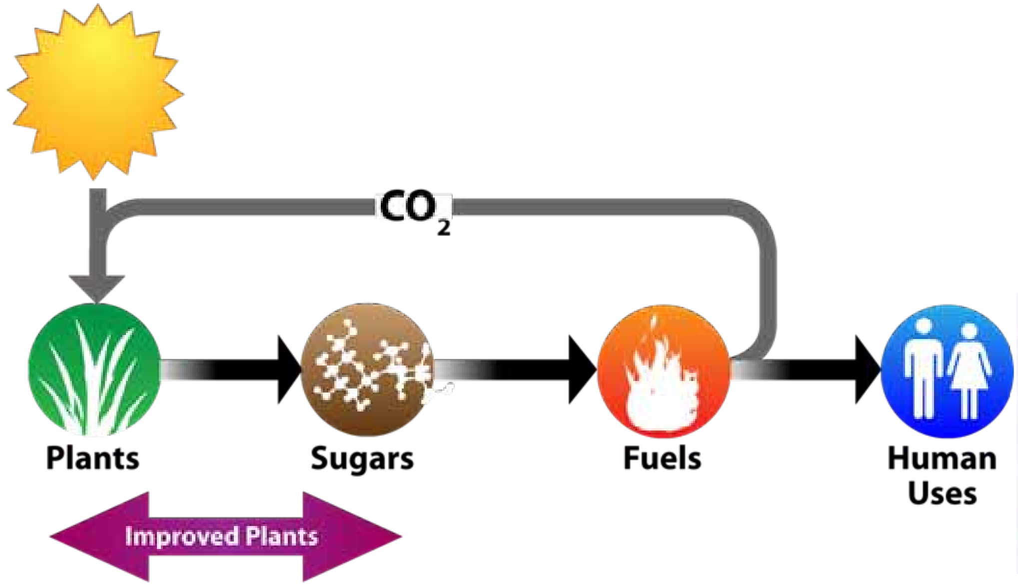
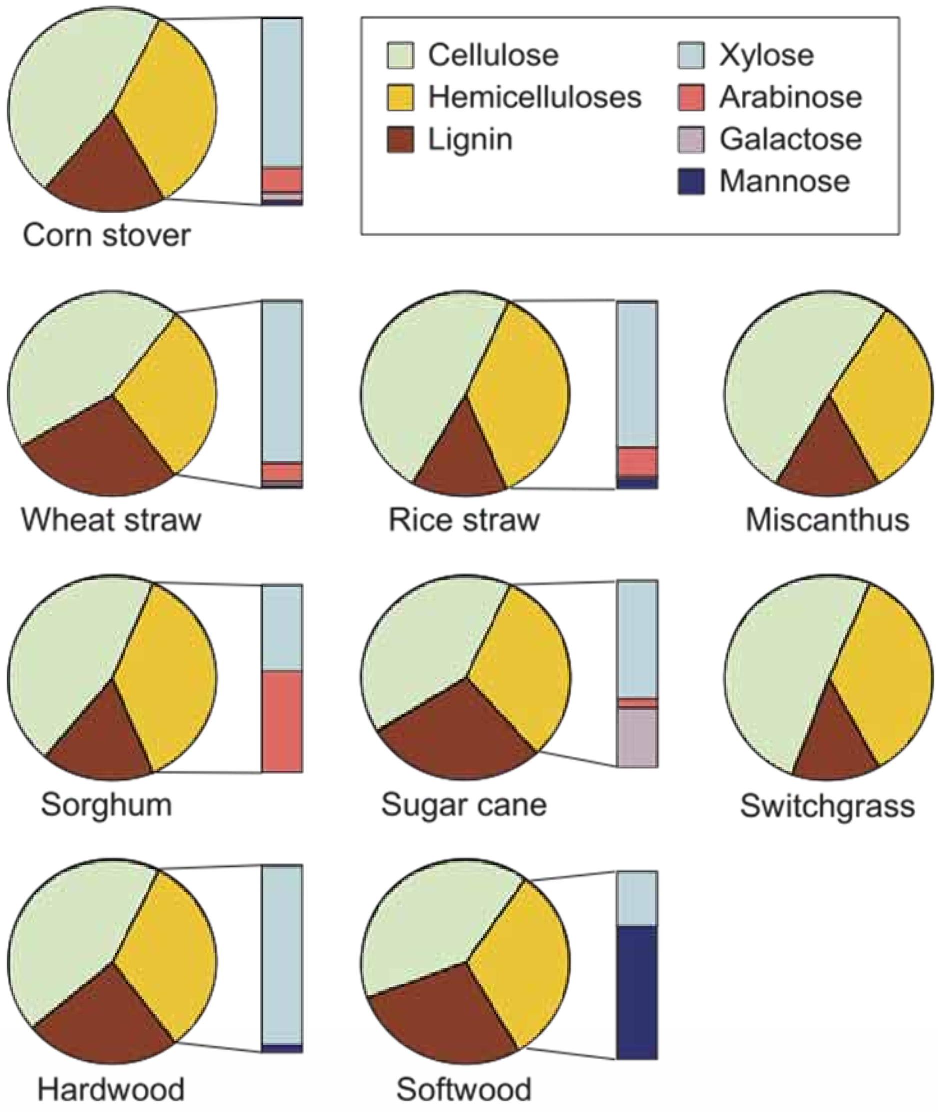


- Biomass plants will need different traits than those traditionally used for food
- Organization/composition of plant cell wall limits sugars for fuel production

Issue	**Approach**
Lignin blocks access to sugars	Lignin "Zips"
Hard to generate fuel from cell wall sugars	Modify hemicelluloses
Do not store other energy polymers	Accumulate oils, starch

Altering Hemicelluloses to Improve Cell Wall Biofuel Traits



Lignin, Cellulose & Hemicelluloses

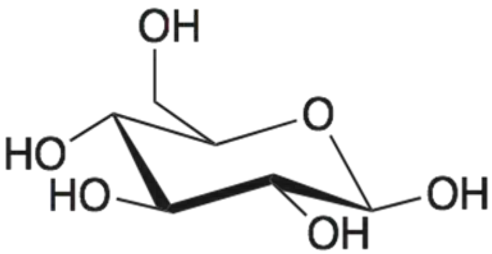
- Composition varies across spectrum of potential biomass plants
- Sugar composition of hemicelluloses vary within a biomass plant
  - Xyloglucan
  - Galactomannan
  - Glucoarabinoxylan

Pauly & Keegstra (2008) Plant J 54: 559-568

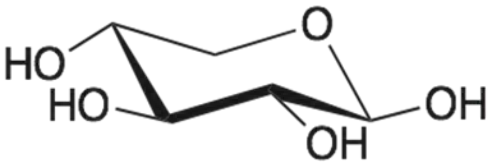
Altering Hemicelluloses to Improve Cell Wall Biofuel Traits

Major sugar monomers in hemicellulose polymer

Glucose	Xylose



$C_6H_{12}O_6$



$C_5H_{10}O_5$

Microbial Fermentation: Good (Glucose) — Poor (Xylose)

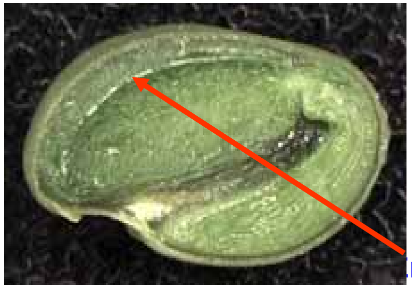
- Alter types of hemicelluloses to provide more hexoses (fermentable C6 sugars)
- Need information on which candidate sugar polymerization enzymes are devoted to which hemicellulose biosynthetic pathways

Dissect Hemicellulose Biosynthesis in Model Plants

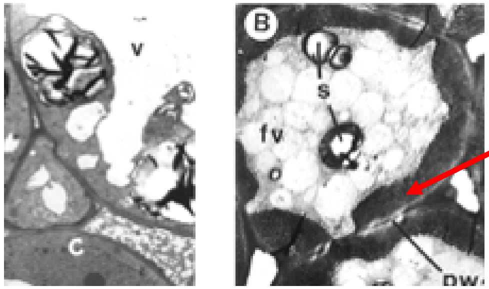
Fenugreek

Psyllium

Nasturtium



Mucilaginous layer
60% Xylan



20 DPA

22 DPA

Cotyledon
30% Xyloglucan

ndosperm
90% Mannan

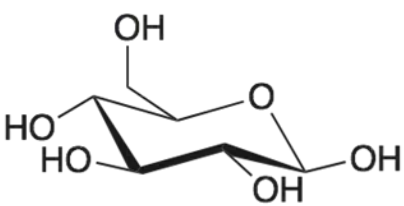
- Profile gene expression in developing seeds rich in specific hemicelluloses
- Production is rapid & occurs before rapid cell growth begins

Keegstra, Pauly, Wilkerson

Altering Hemicelluloses to Improve Cell Wall Biofuel Traits

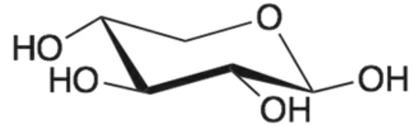
Major sugar monomers in hemicellulose polymer

Glucose



$C_6H_{12}O_6$

Xylose

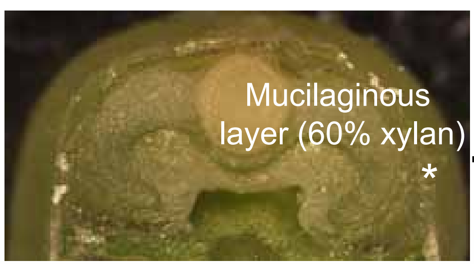


$C_5H_{10}O_5$

Microbial Fermentation: Good (glucose) / Poor (xylose)

1. Dissect xylan polymer synthesis by transcript profiling of "Xylose-enriched" (Psyllium) seedlings

→



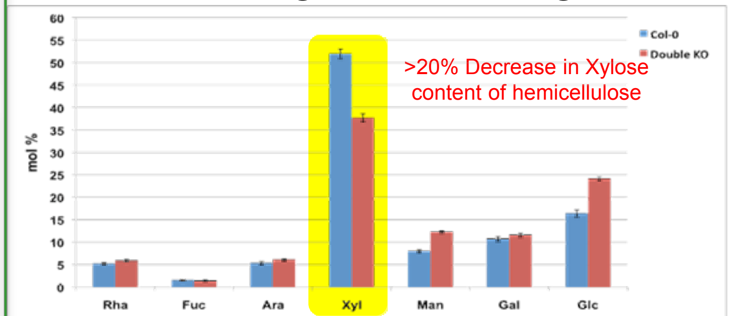
Transverse section of seed

→ Micro-dissection to prepare tissue-enriched RNAs

2. Sequence EST libraries to identify candidate genes (JGI)

Psyllium	Stage	6 DPA	8 DPA	10 DPA	12 DPA
(Xylan)	# ESTs (**897K**)	144,368	297,723	252,232	202,403

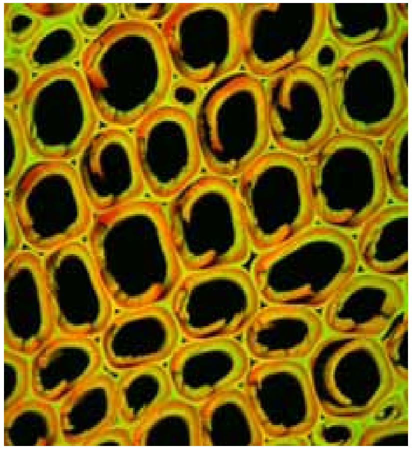
3. Test (confirm) function by inactivating candidate gene**



**patents applied for

4. Analysis of xylose & other hemicellulose pathways moving into potential bioenergy plants

Improving Energy Traits of Plant Biomass



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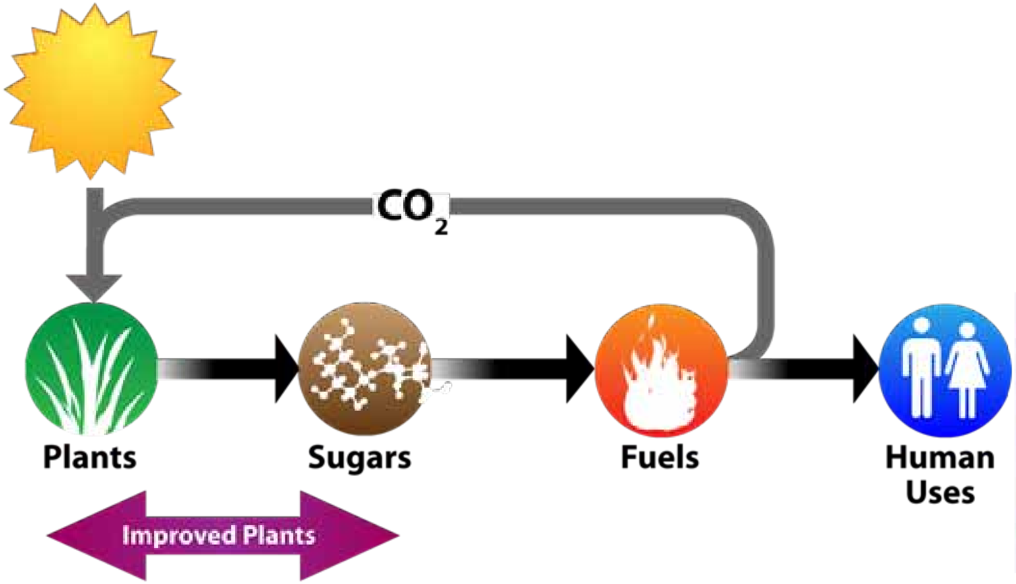

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Issue	**Approach**
Lignin blocks access to sugars	Lignin "Zips"
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Direct Plant Carbon into Other Energetic Polymers

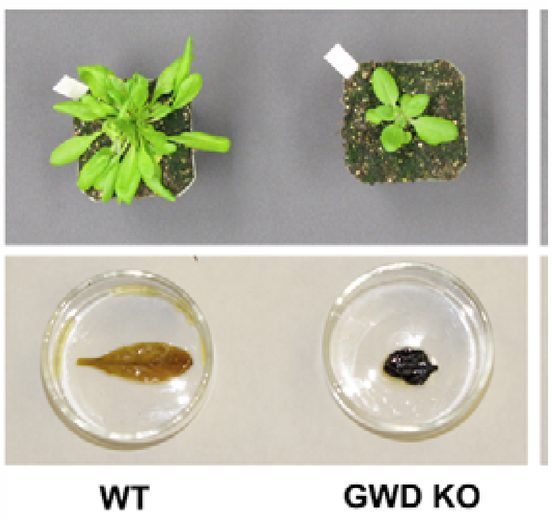
Starch accumulation in growing (vegetative) plant tissue

- Can be stored at up to 50% dry weight
- Metabolically inert polymer composed of easily fermented glucose monomers
- No energetic transport penalty since made in chloroplast (site of photosynthesis)
- Vegetative storage does not compete directly with food supply

Approach to accumulate starch in vegetative tissue

- Lack of Glucan Water Dikinase (GWD) causes severe starch excess

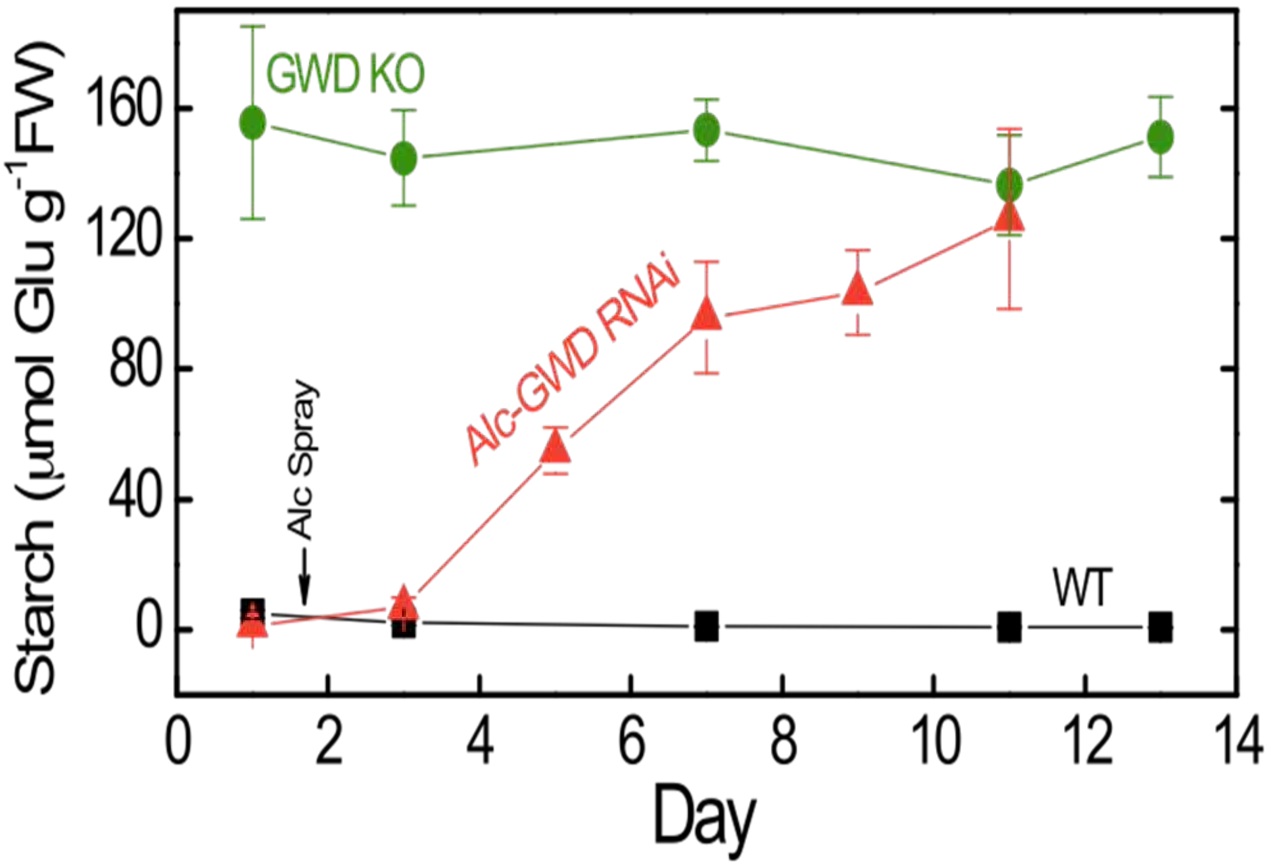
Stain starch with Iodine (blue)



Yield penalty of starch accumulation in GWD KO leaves

Direct Plant Carbon into Other Energetic Polymers

- **Inducible starch accumulation in vegetative (growing) plant tissue****



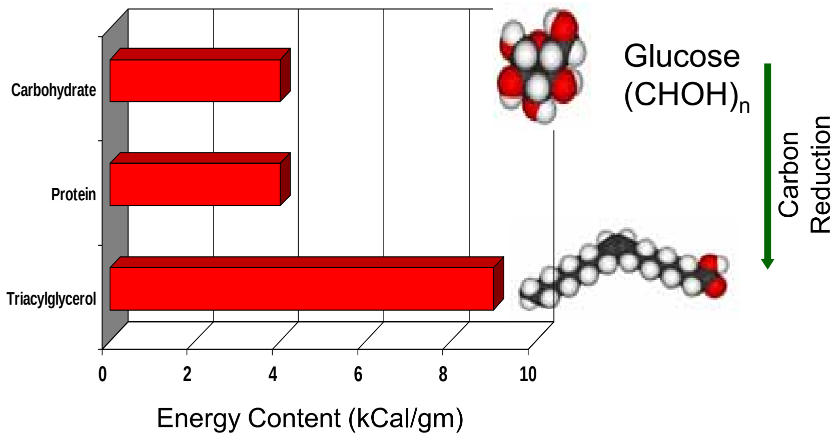
Tunable starch accumulation (20% of dry weight within 1 week)

Sharkey

**patents applied for

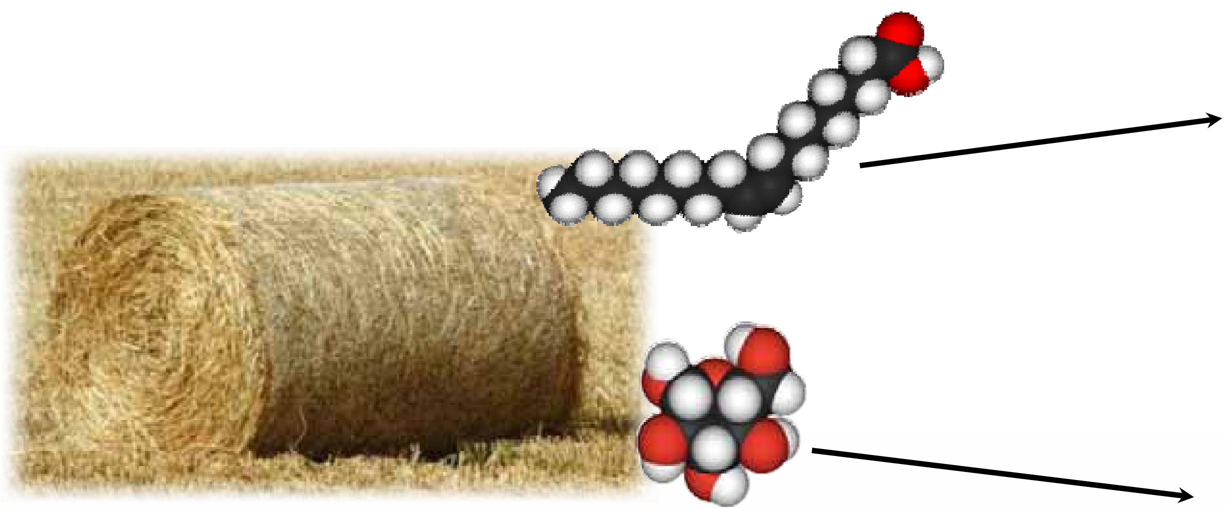
Direct Plant Carbon into Other Energetic Polymers

- **Oil accumulation in growing (vegetative) plant tissue****



- Oils are the most energy-rich form of carbon polymer
- Use of plant oils as fuel does not require fermentation
- Vegetative cell oil does not compete directly with food supply

- 20% oil content would **~double** the energy content available/acre for liquid fuels



3 tons oil
(~3000 L biodiesel)
~113 GJ energy

12 tons lignocellulose
(~5000 L ethanol)
~117 GJ energy

Enhancing Energy Density in Biomass

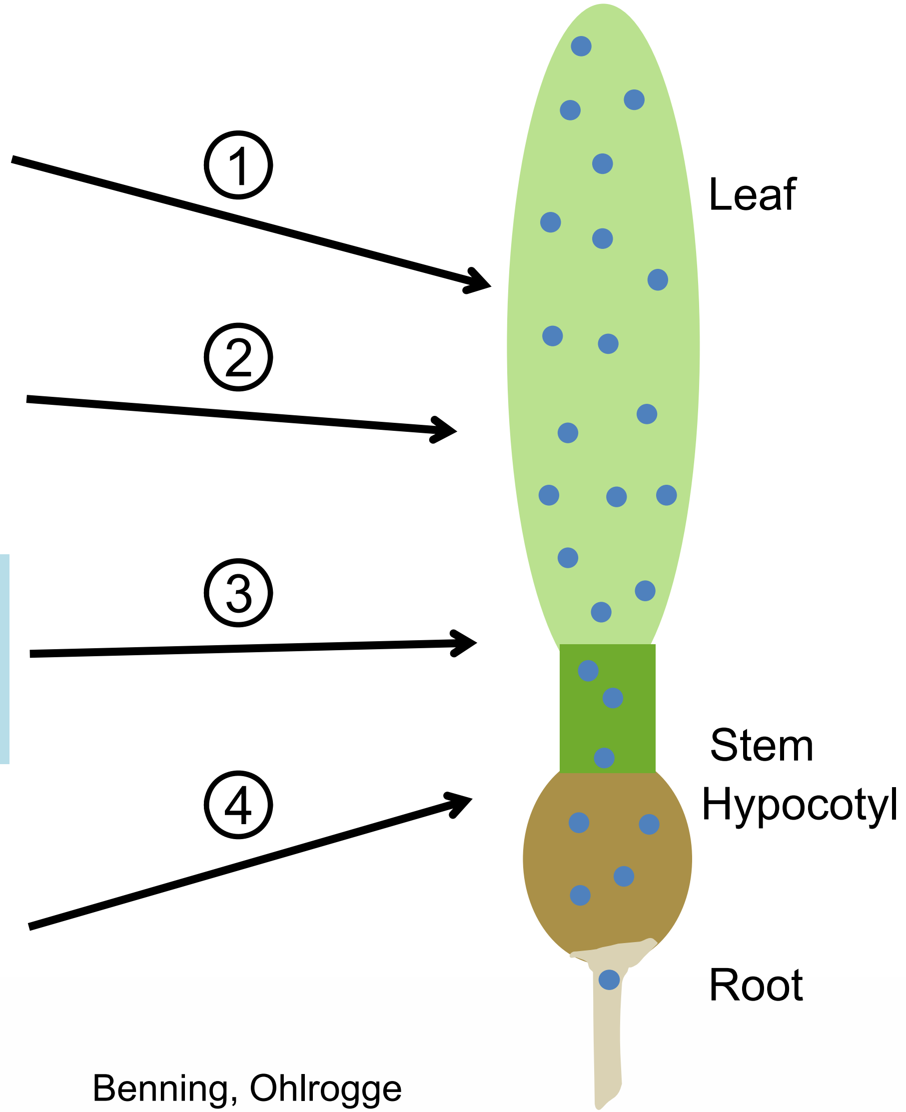
- Strategies to Accumulate Oil in Vegetative Tissues

Enhance Oil Biosynthesis
(Over-express DGAT)

Decrease Oil Breakdown
(Inhibit CTS)

Divert Carbohydrates into Oil
Starch (↓ AGPase RNAi)
Glycolysis (↑ WRI1)
Fatty Acids (↑WRI1)

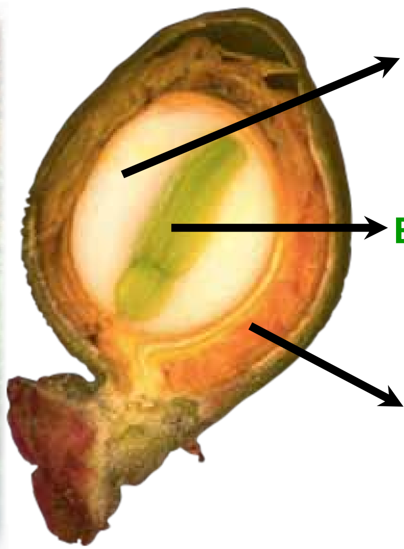
Make Non-degradable Oils
(Wax Synthesis)



Benning, Ohlrogge

Enhancing Energy Density of Biomass

- Discovery of an acetyltransferase that synthesizes acetylglycerols (***Ac*TAG**)
- Burning bush seeds contain 50% oil by weight; >95% of oil in endosperm is acetyl-triacylglycerols (***AcTAG***)



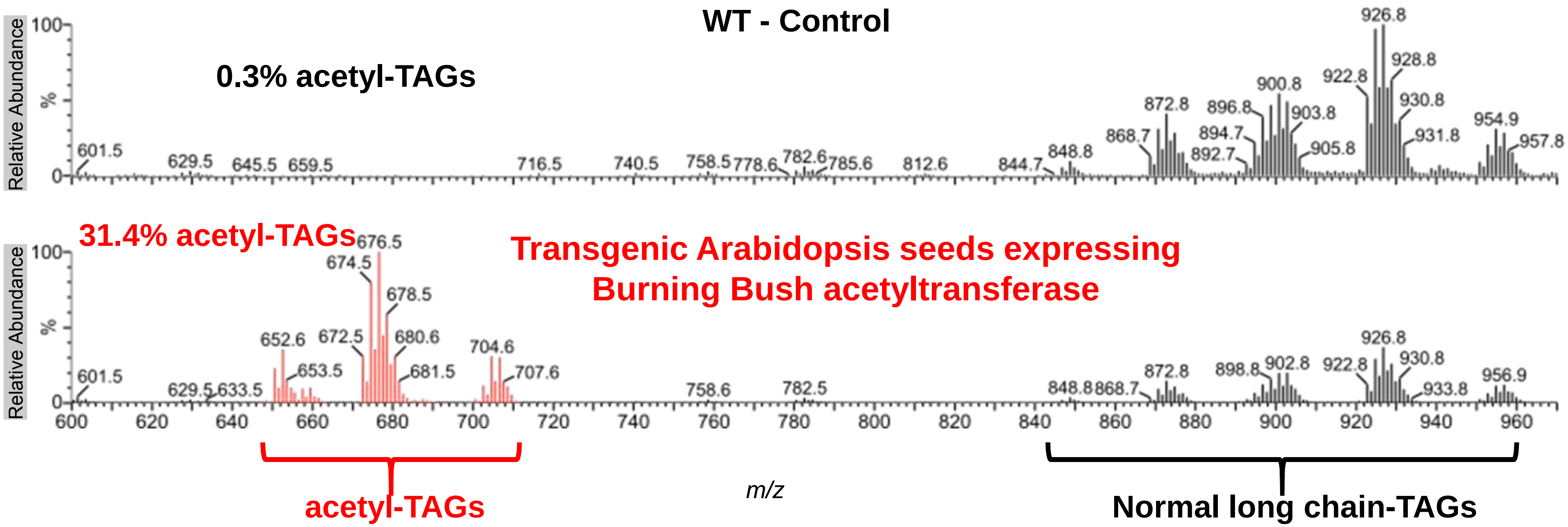
AcTAG

$CH_2OCO(CH_2)_7CH{=}CH(CH_2)_7CH_3$
|
$CHOCO(CH_2)_7CH{=}CH(CH_2)_7CH_3$
|
CH_2OCOCH_3

- Analysis of large number of ESTs across seed body allows for quantitative comparisons between tissue types

Enhancing Energy Density of Biomass

- Production of Acetyl-TAG (Low-viscosity Oil) in Transgenic Plants**



- Candidate acyltransferase genes selected based on tissue-specific EST expression pattern
- Candidates screened for acetyl-TAG formation when placed in yeast
- When placed in Arabidopsis oil produced in seeds (ESI-MS)
- Experiments in progress to analyze acetyl-TAG production in other tissues

Ohlrogge

**patents applied for

Enhancing Energy Density in Biomass

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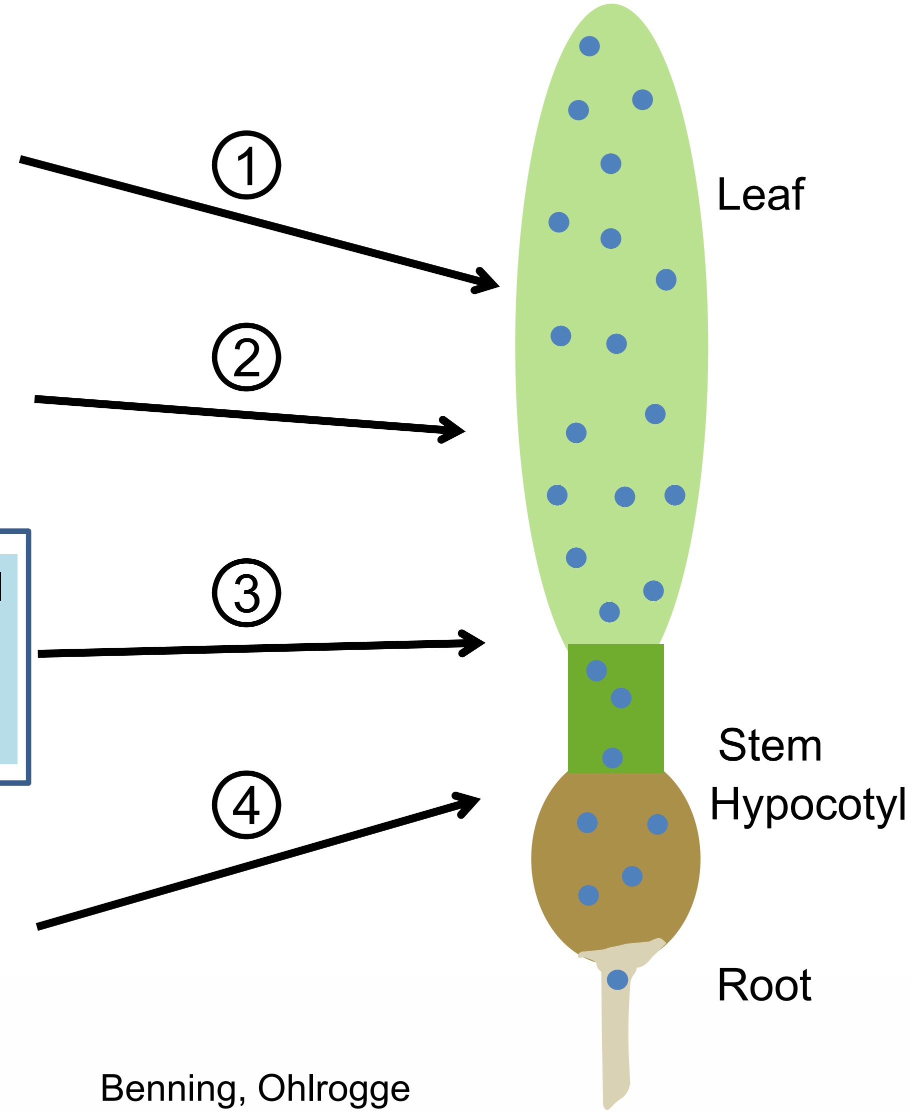
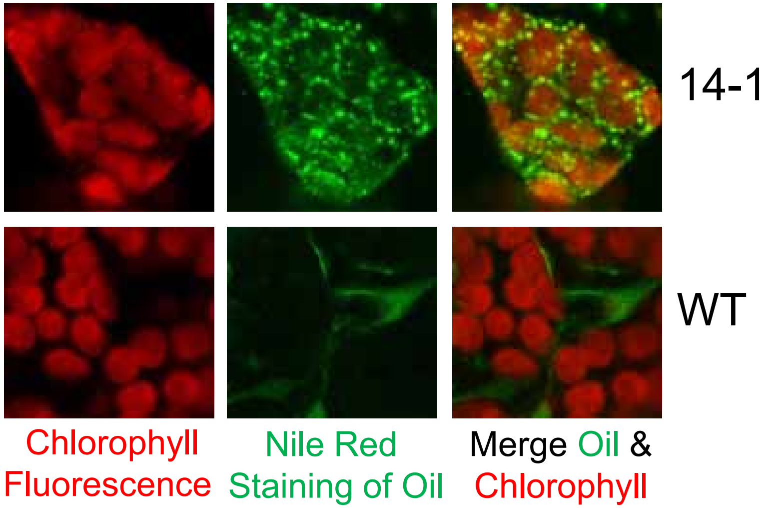


Benning, Ohlrogge

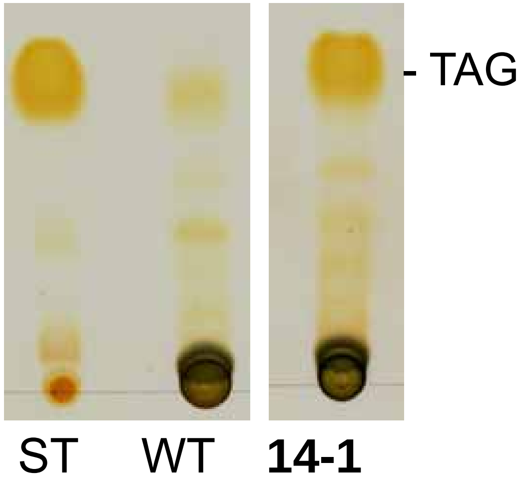
Enhancing Energy Density in Biomass

- Diverting carbon into oil by adjusting both AGPase & WRI1

Confocal Microscopy



Thin Layer Chromatography

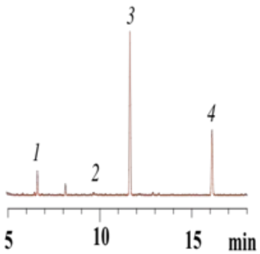


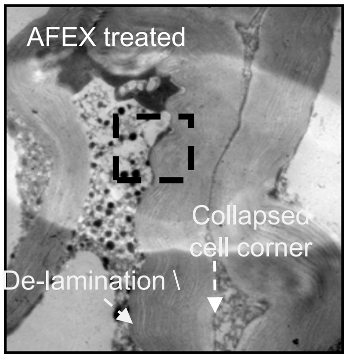
- Fatty acid content of Arabidopsis 14-1 leaves ~2-3% of dry weight (~3X increase)

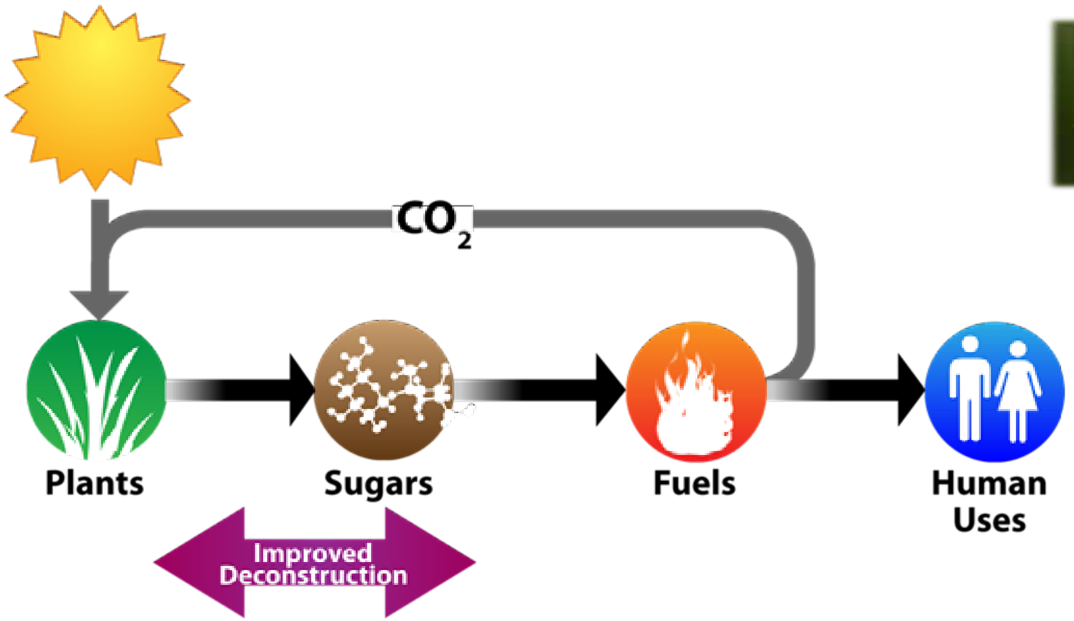
Benning

**patents applied for

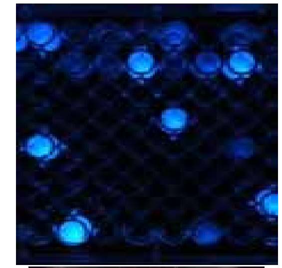
Improving Plant Biomass Deconstruction

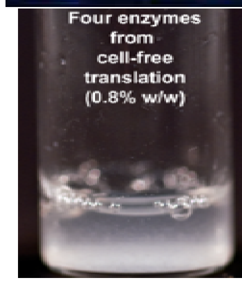






Leaf cutter ants

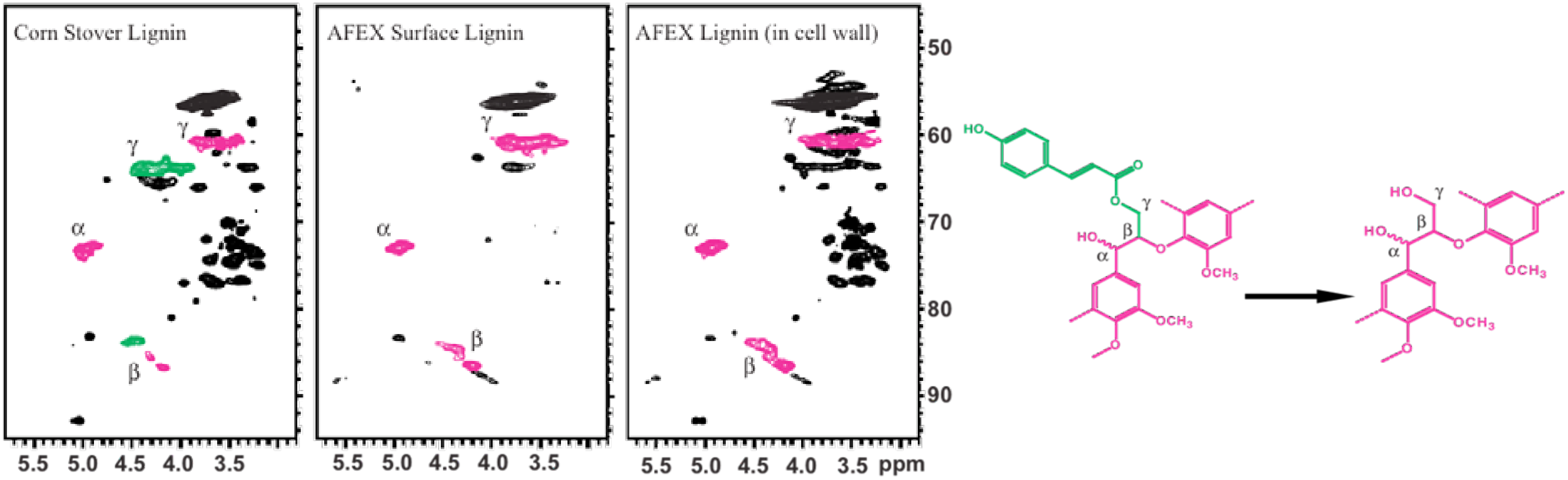




- Pretreatments expose cell wall sugar polymers
- New & improved enzymes cut sugar polymers into soluble units

Issue	Approach
Lignin blocks access to sugars	Dissect lignin destruction
Identify, test & improve activity of enzymes	Enzyme pipeline
Sugar release requires pretreatment	Methods development

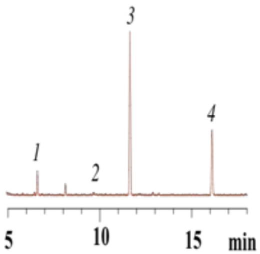
Mapping Cell Wall Changes by Magnetic Imaging

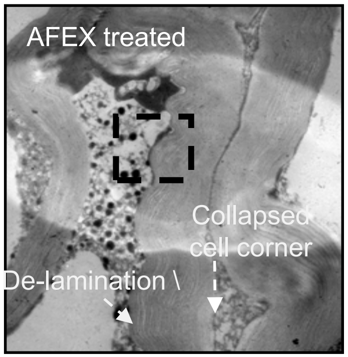


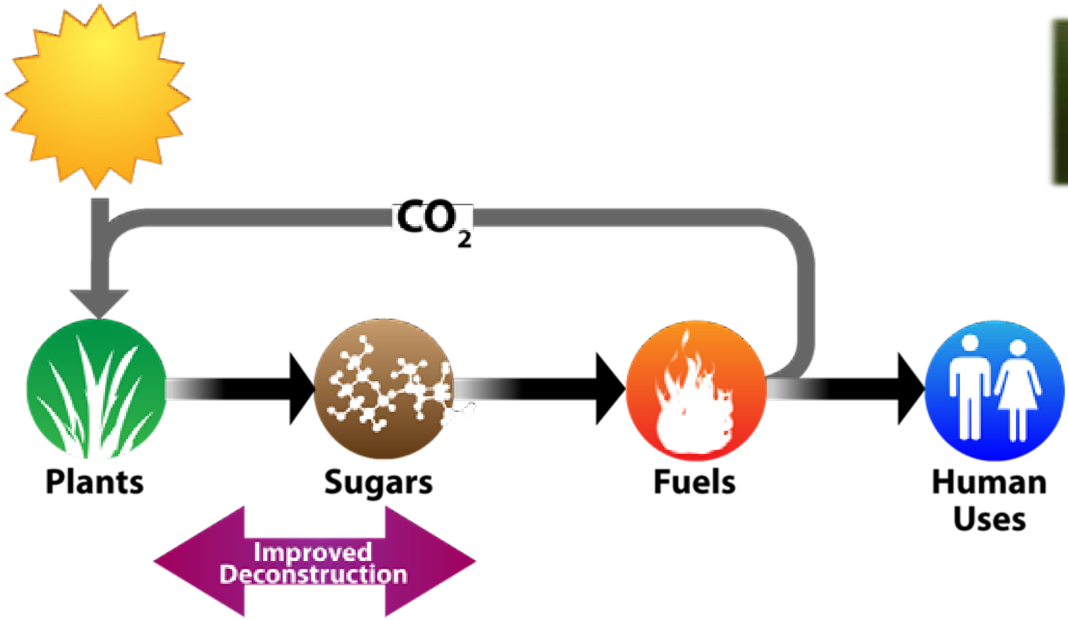
- Effects of AFEX (ammonia) pre-treament
  - Overall structure of lignin polymer unchanged
  - Lignin esters (*p*-coumarates) removed
  - Cleaves cross links that otherwise block enzymatic sugar release
  - ARRA-funded core facility to provide routine capability across BRCs

Ralph, Dale

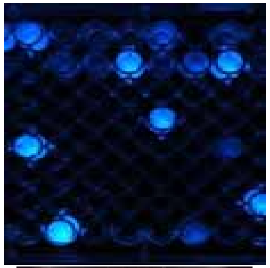
Improving Plant Biomass Deconstruction

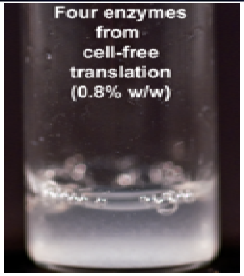






Leaf cutter ants



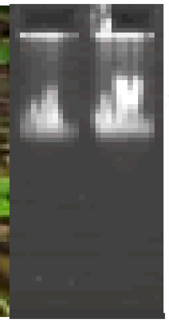


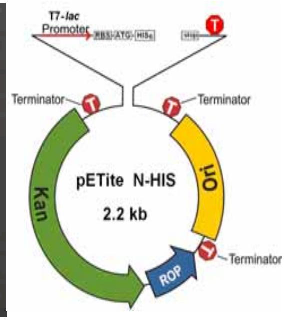
- Pretreatments expose cell wall sugar polymers
- New & improved enzymes cut sugar polymers into soluble units

Issue	Approach
Lignin blocks access to sugars	Dissect lignin destruction
Identify, test & improve activity of enzymes	Enzyme pipeline
Sugar release requires pretreatment	Methods development

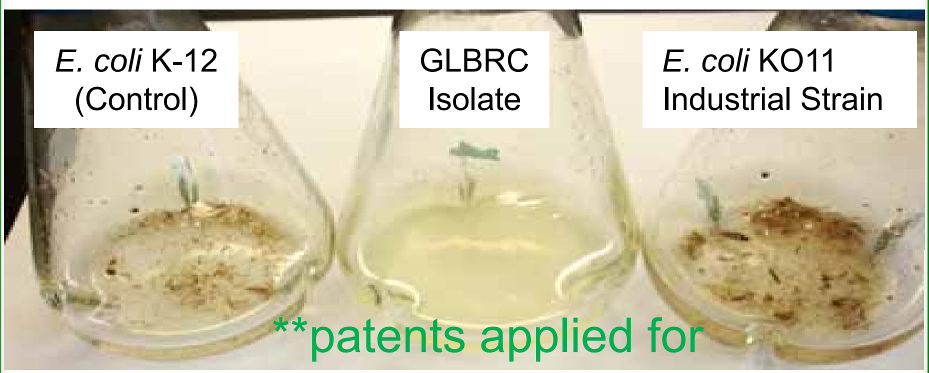
Combinatorial Enzyme Discovery & Analysis Pipeline

Screen ecosystems, microbes & gene products for sugar release from pretreated biomass (Lucigen, Currie)**





Newly-identified bacteria degrade sugars in AFEX-treated corn stover**



**patents applied for

- > 2000 cellulolytic microbes isolated from environments with high rates of lignocellulose utilization (Currie)

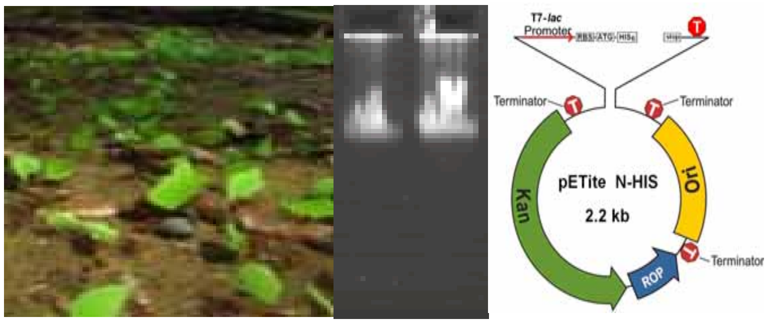
Leaf cutter ants

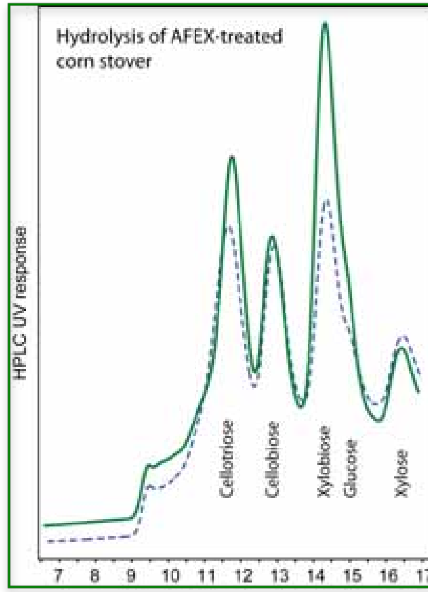
Fungus-growing termites

Mountain pine beetle

Combinatorial Enzyme Discovery & Analysis Pipeline

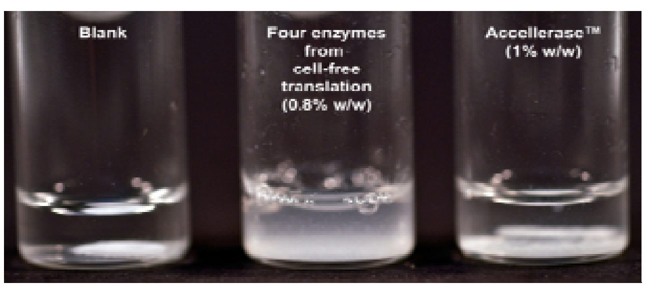
Screen ecosystems, microbes & gene products for sugar release from pretreated biomass**



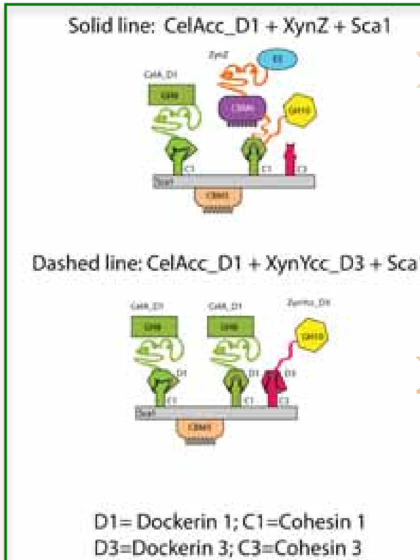


- Test sugar release from cellulose substrates by single enzymes & mixtures *in vitro* (Fox, Walton, Dale)**

Cell-free enzyme synthesis allows facile combinatorial analysis of enzyme mixtures (Fox)**



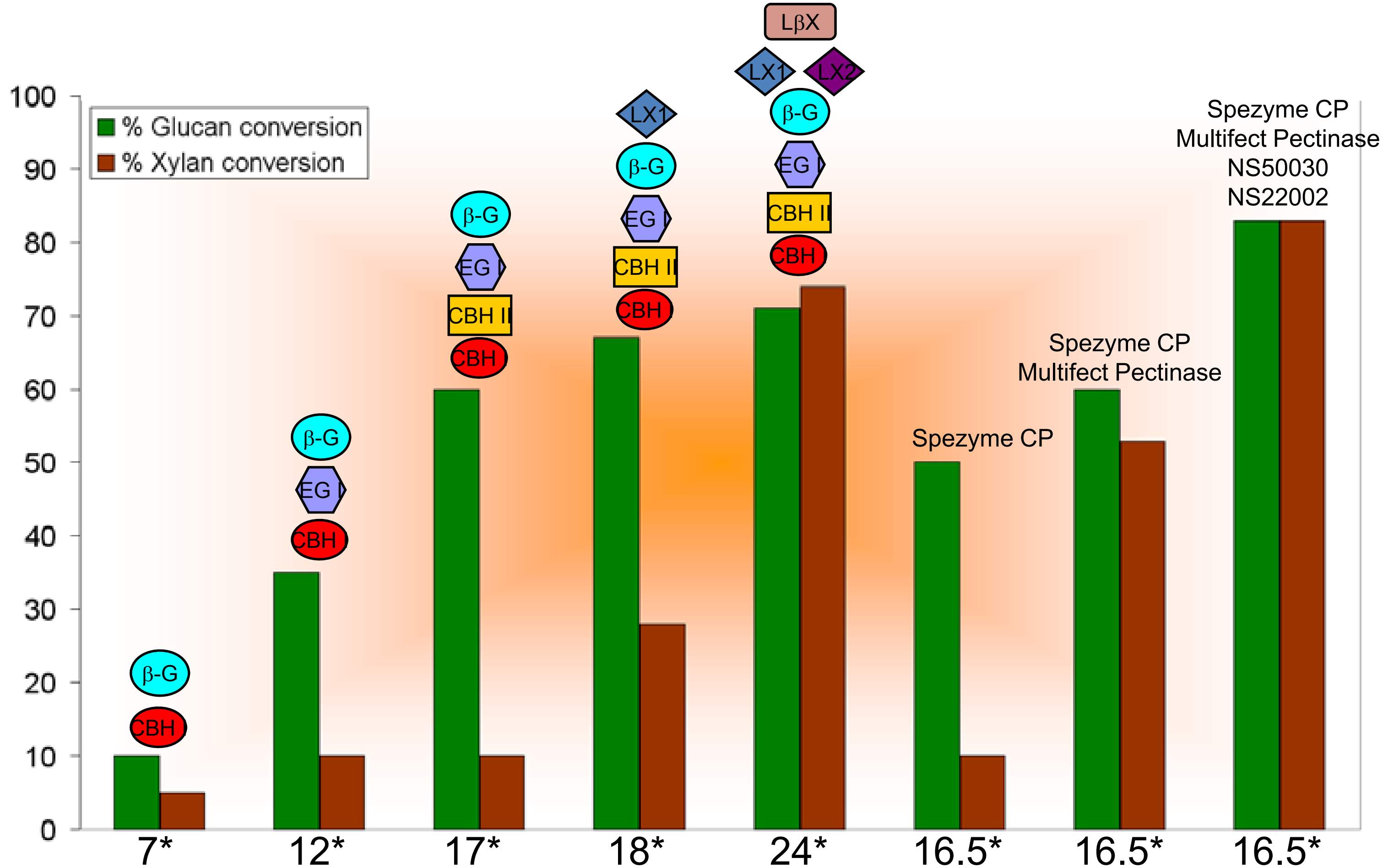
**patents applied for



- Test sugar release using different enzyme scaffolds or architectures *in vitro* (Fox)**
- Promising candidates moved to microbial expression tests

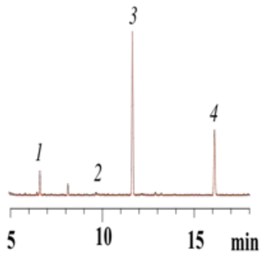
Combinatorial Enzyme Discovery & Analysis Pipeline

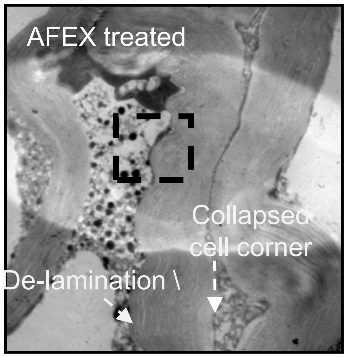
- Purified enzymes compete with commercial mixtures for sugar release from AFEX-treated corn stover (Walton, Dale, Lucigen, Fox)

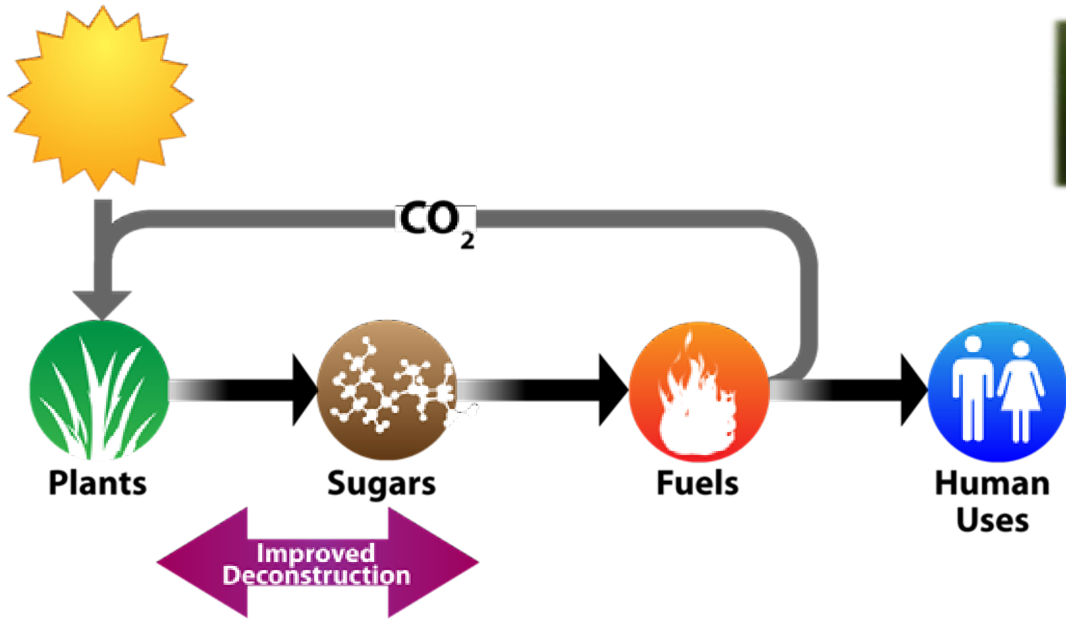


*Protein Loading (mg/g glucan), 24 h hydrolysis of milled AFEX-treated corn stover (milled)

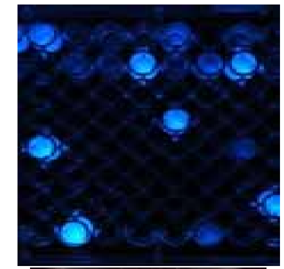
Improving Plant Biomass Deconstruction

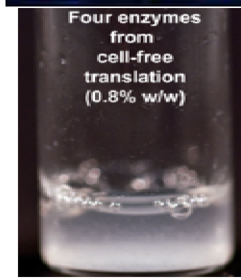






Leaf cutter ants

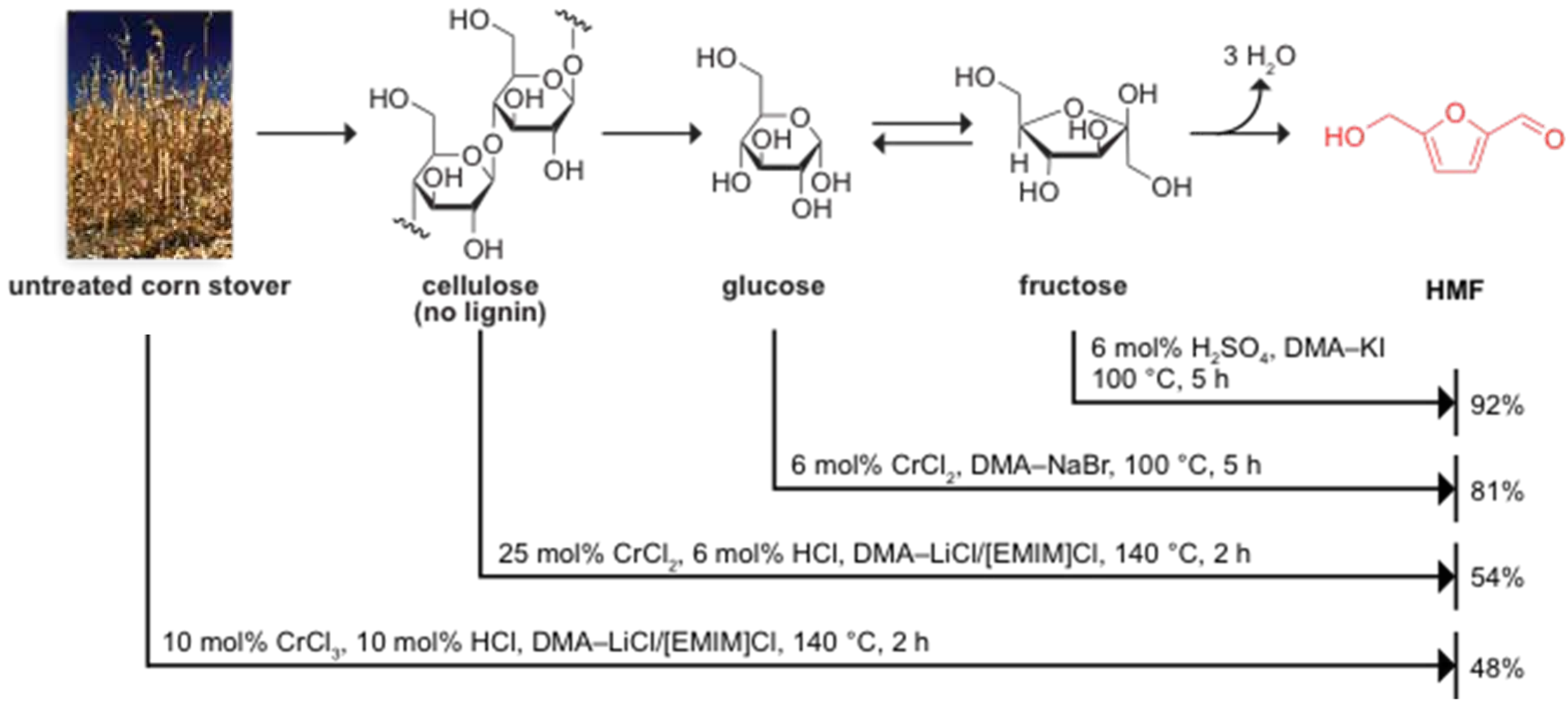




- Pretreatments expose cell wall sugar polymers
- New & improved enzymes cut sugar polymers into soluble units

Issue	**Approach**
Lignin blocks access to sugars	Dissect lignin destruction
Identify, test & improve activity of enzymes	Enzyme pipeline
Sugar release requires pretreatment	Methods development

Fuel Precursors (HMF) from Crude Plant Biomass in 1-step



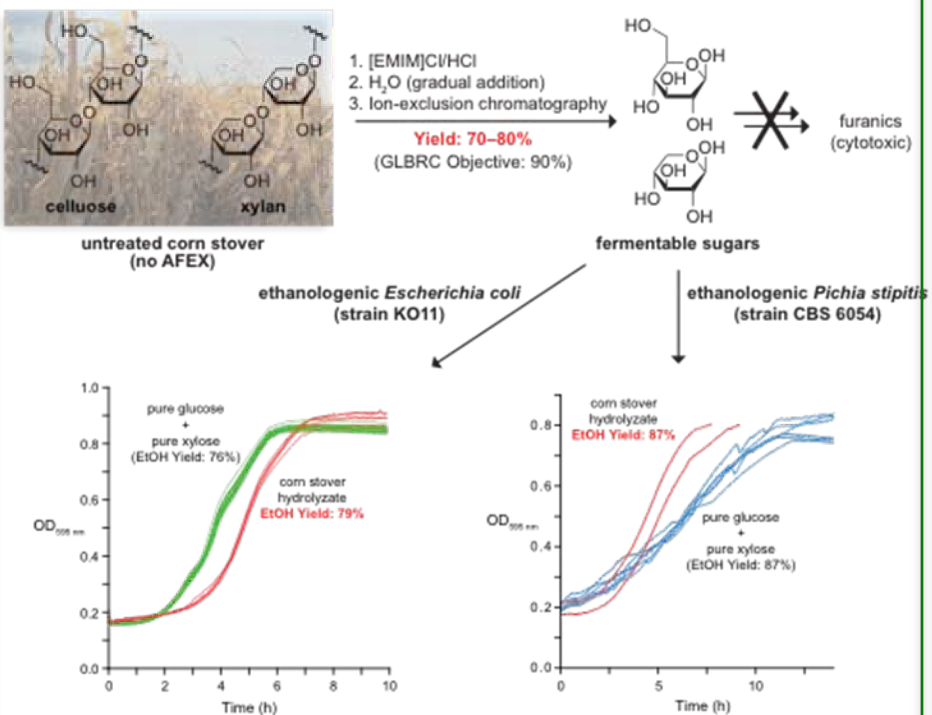
1. Yields do not depend on pretreatment
2. Yields do not depend on presence/absence of lignin
3. Mechanism of HMF formation allows rational design of catalysts with less toxicity

Binder, J. & R. Raines J. Am. Chem. Soc. 2009, *131*, 1979**

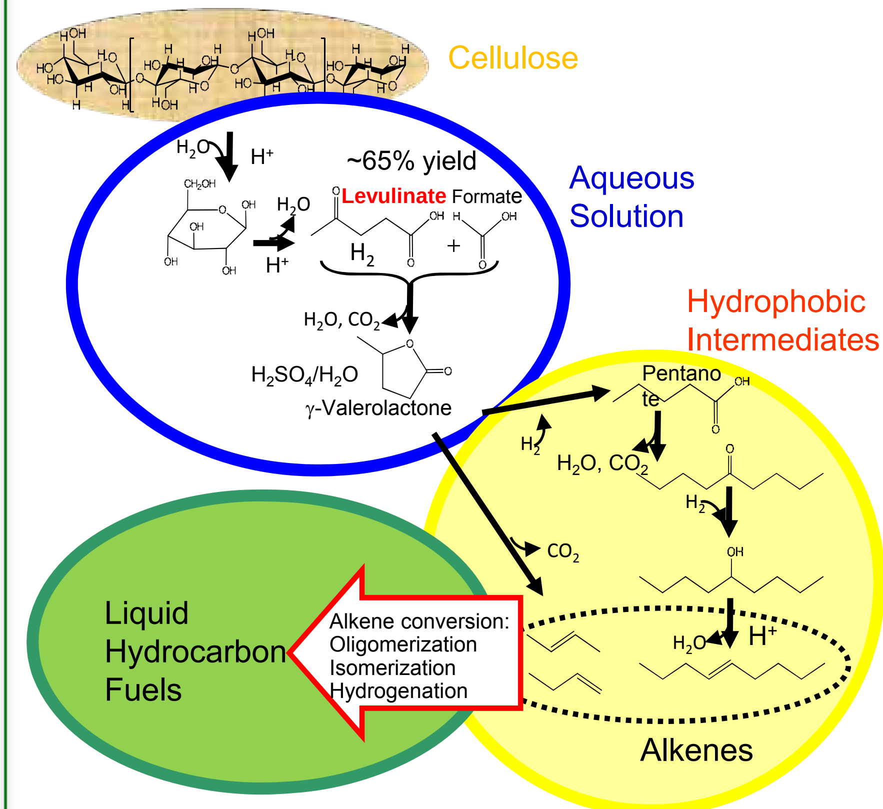
**patents applied for

Improved Chemistries for Cellulosic Fuel Production

One-step release of fermentable sugars from lignocellulose (Raines)**

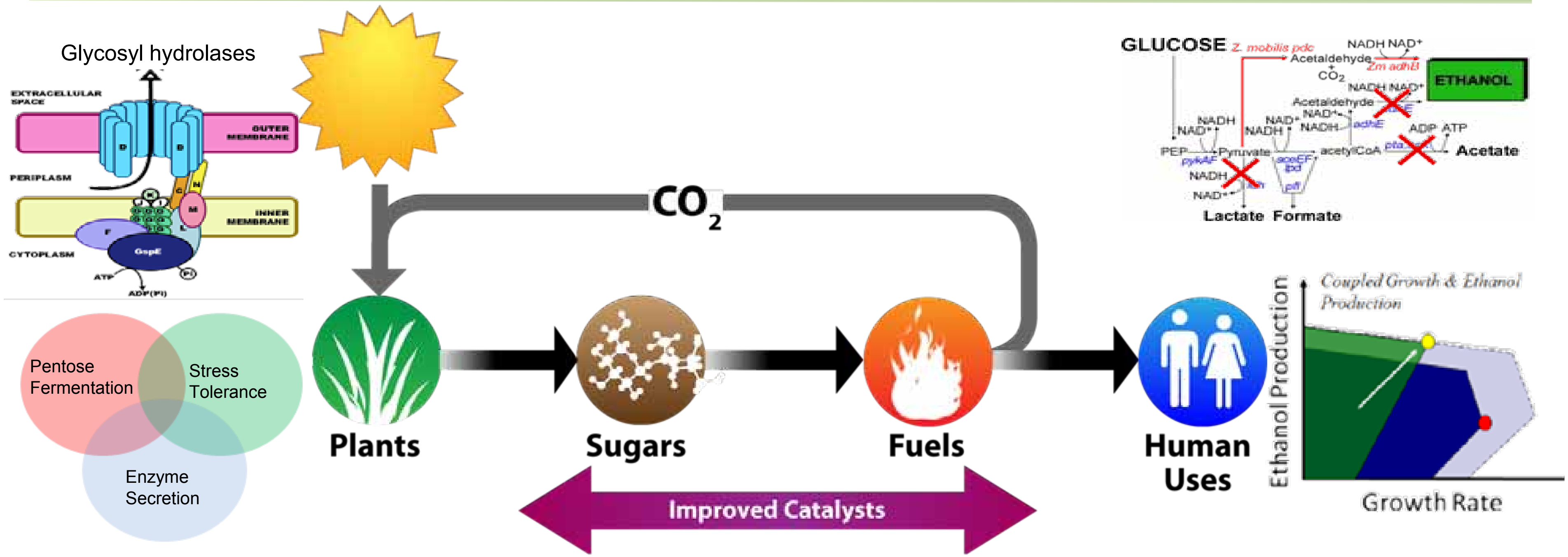


Levulinate as an improved (less volatile & more stable) catalytic intermediate (Dumesic)**



**patents applied for

Improving Catalysts for Fuel Production

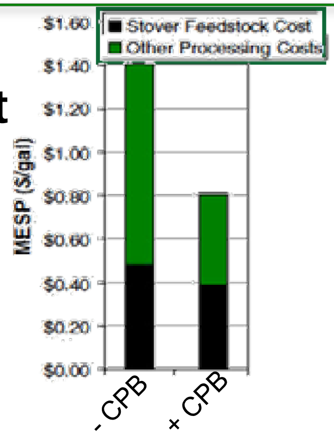


- Accommodate chemical mixtures (sugars, toxins, etc) in cellulosic feedstocks
- Microbial & chemical activities that consolidate production of ethanol, other fuels

Issue	**Approach**
Complex sugar stream	Robust catalytic processes
Consolidated fuel production	Optimize use of cellulosic feedstocks
Next generation fuel microbes	Strategic building blocks, EtOH & others

Consolidated Biofuels Platform (CBP)

CBP lowers $$/gallon cost of biofuels
Sendich et al. 2008 *Bioresource Tech*.99:8429



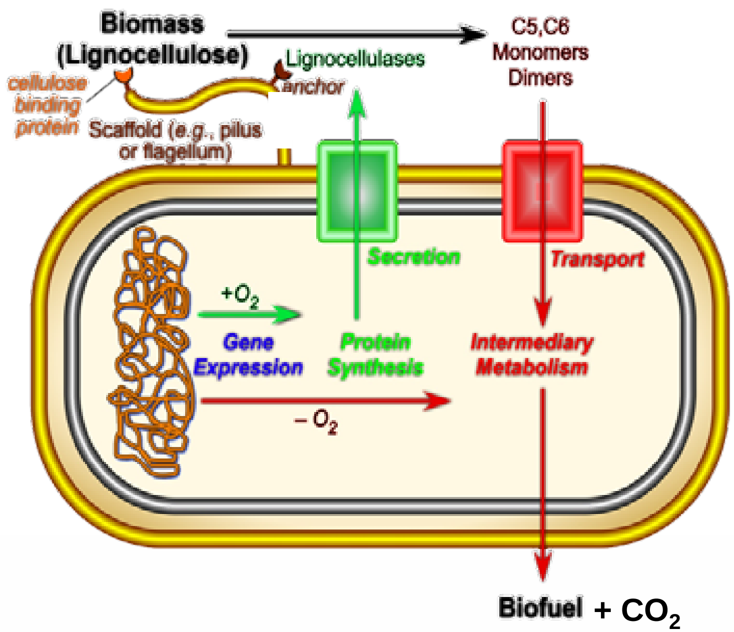
Optimize Cellular Network for CBP

Cost to make 360 aa protein: 1500 ATP

Sugars

$+O_2$ → $6\ CO_2 + 6\ H_2O$ + ~30 ATP **(~2850 kJ)**

$-O_2$ → 2 EtOH + CO_2 + 2 ATP **(~118 kJ)**



Two-step CBP refinery strategy

A. Aerobic growth ($+O_2$): Optimize production of extracellular enzymes, & biomass anchors

B. Anaerobic growth ($-O_2$): Optimize production of enzymes, transporters, precursors & fuels

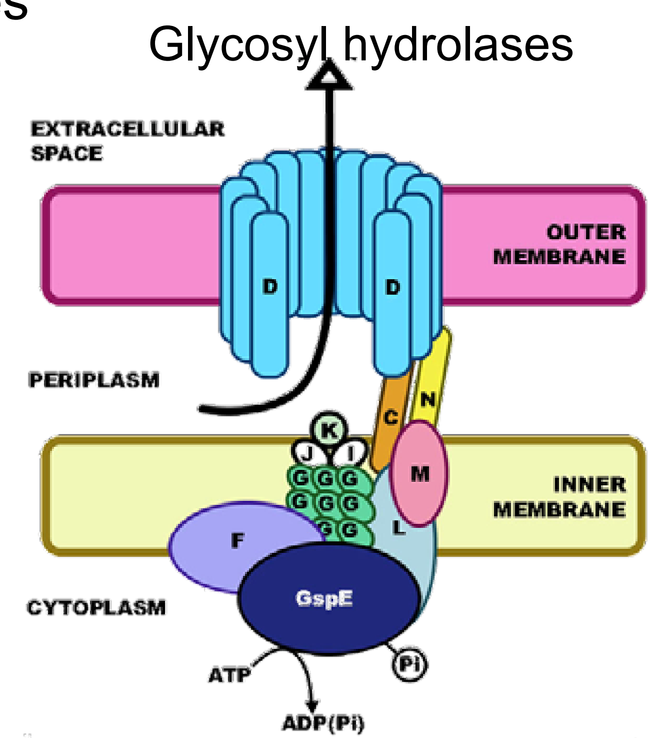
Major Microbes
Escherichia coli
Saccharomyces cerevisiae
(Biotech workhorses & best models)
+ ones to convert CO_2 into co-product(s)

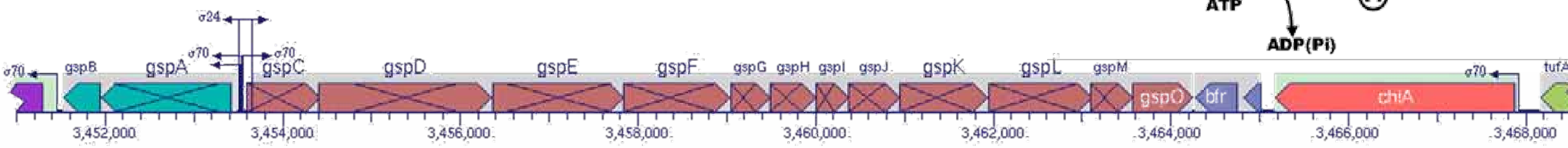
Producing lignocellulases in Gram-negative bacteria

1. Identify relatives of *E. coli* that degrade AFEX-corn stover efficiently
   - Entereobacterial phytopathogens
     (Nicole Perna, Jeremy Glasner)
   - γ-proteobacteria from leaf-cutter ant colonies
     (Cameron Currie)

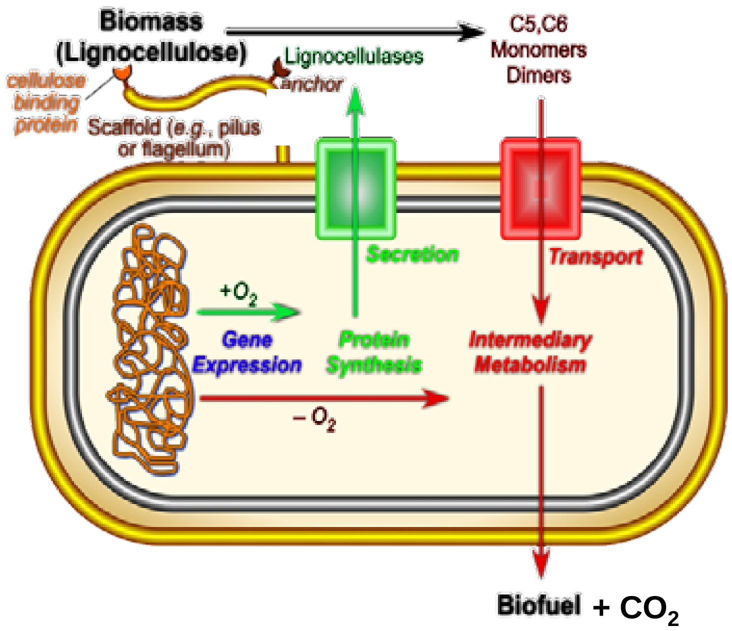
2. Transplant secretion systems (type 2 SS) & libraries of lignocellulase genes to *E. coli*
   - T2SS excrete glycosyl hydrolases
   - T2SS is "silenced" in *E. coli* K-12[1]
   - T2SS can be transplanted[2]

[1]Francetic et al. EMBO J. 2000 19:6697
[2]He et al. PNAS 1991 88:1079





Consolidated Biofuels Platform (CBP)



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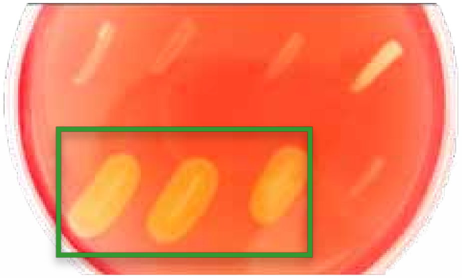
Saccharomyces cerevisiae

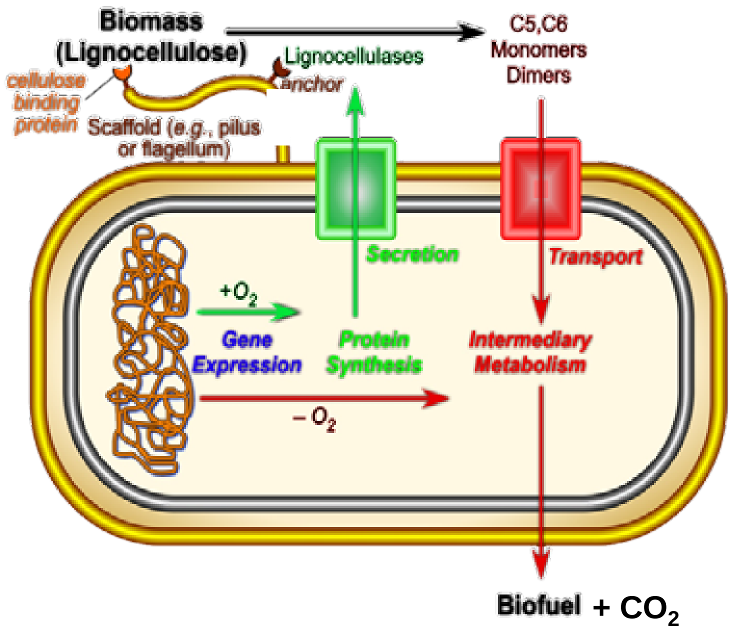
(Biotech workhorses & best models)

+ ones to convert CO_2 into co-product(s)

Enzyme secretion improves *E. coli* cellulose hydrolysis



Improving Microbes for Fuel Production



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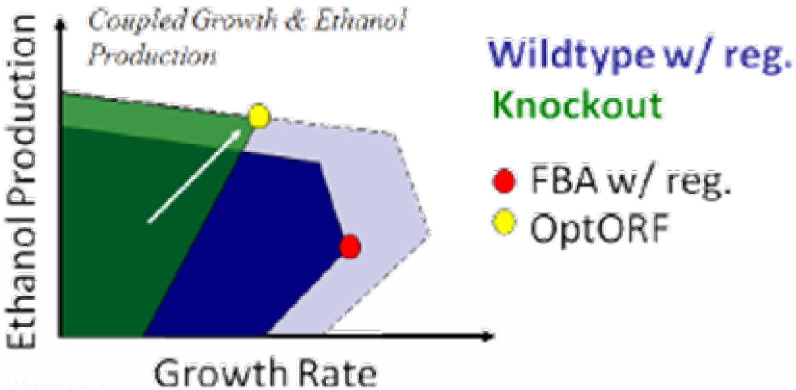
Knockout Production Capabilities

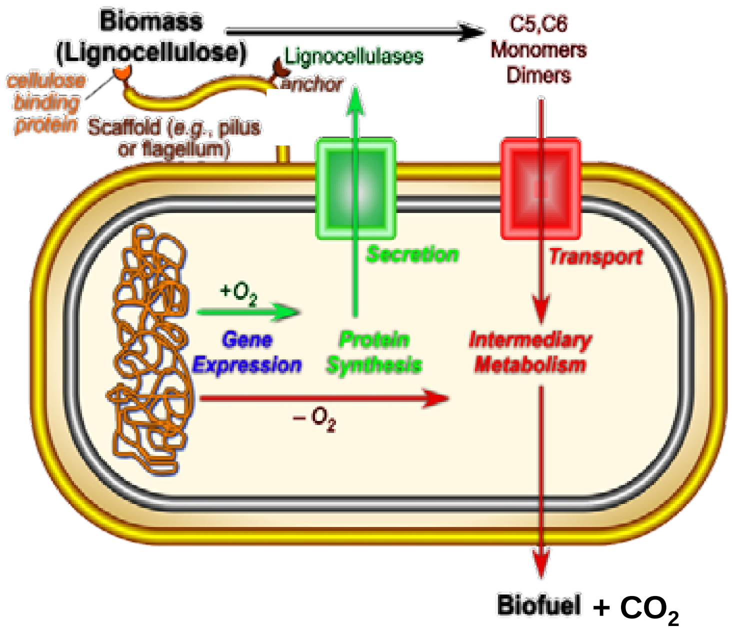


OptORF Approach (*Reed*)

- Simple genetic strategies are identified.
- Accounts for transcriptional circuits
- Identifies metabolic & regulatory candidates

Couple OptORF predictions with chemostats, strain improvement & omics analysis

Improving Microbes for Fuel Production



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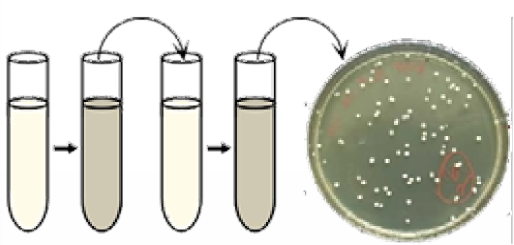
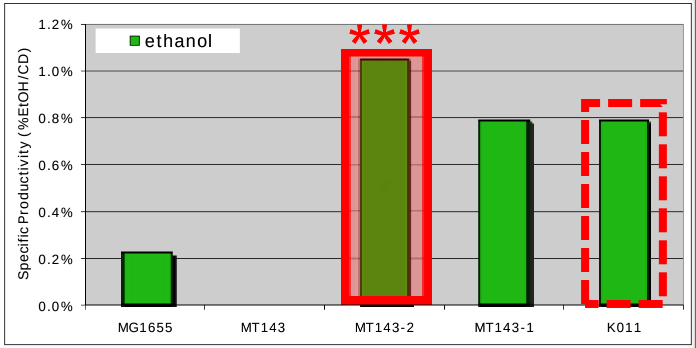
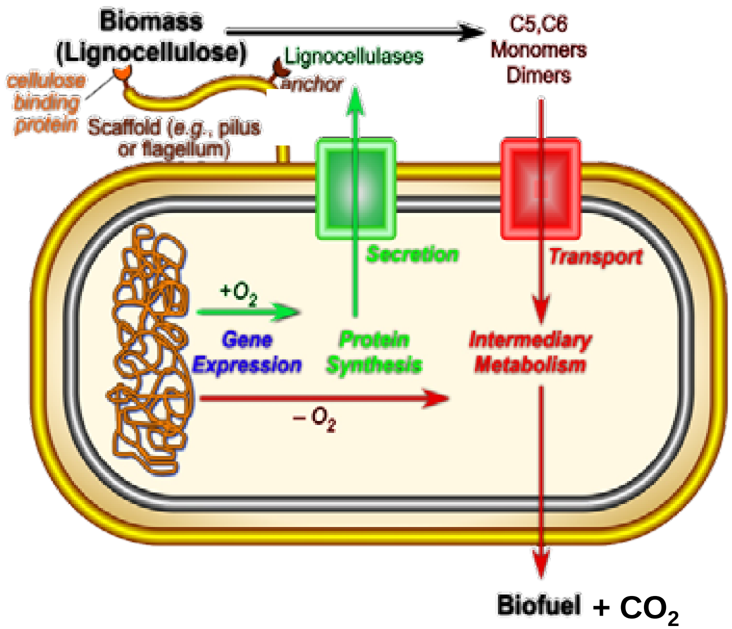
Optimization of anaerobic network (MT143-2) improves fuel (EtOH) production by industrial *E. coli* strain (KO11)





- Some lesions improve performance with single (glucose, xylose) or multiple sugars

Improving Microbes for Fuel Production



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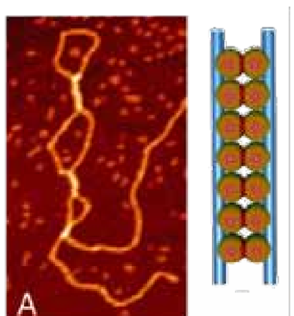
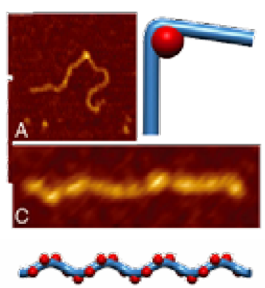
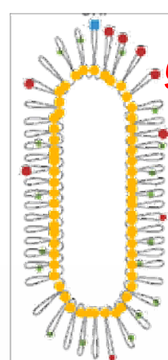
Loops in microbial genomes could enhance anaerobic gene expression**



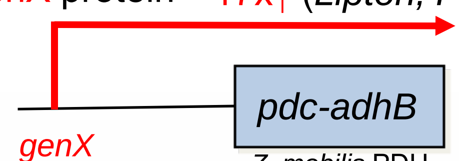
Hu-"silenced" regions



Hu-"activated" loops



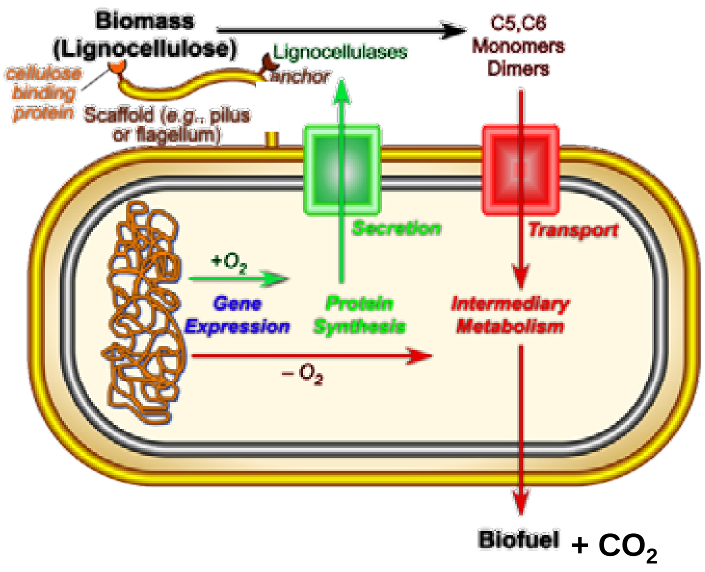
	Fold change $-O_2$
genX Start Site	31x↑ (with JGI)
genX mRNA	40x↑
GenX protein	17x↑ (*Lipton, PNNL*)



Z. mobilis PDH & ADH genes

**patents applied for

Improving Microbes for Fuel Production



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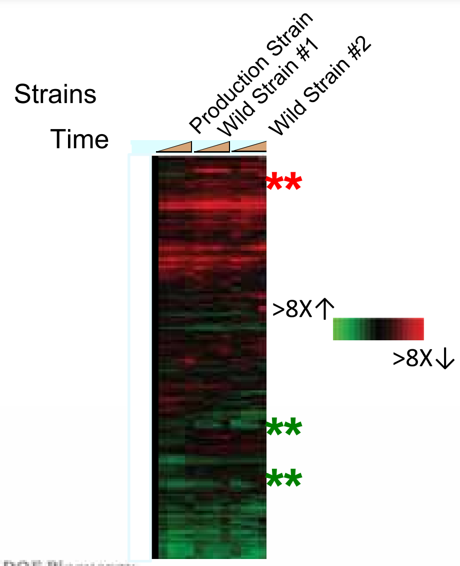
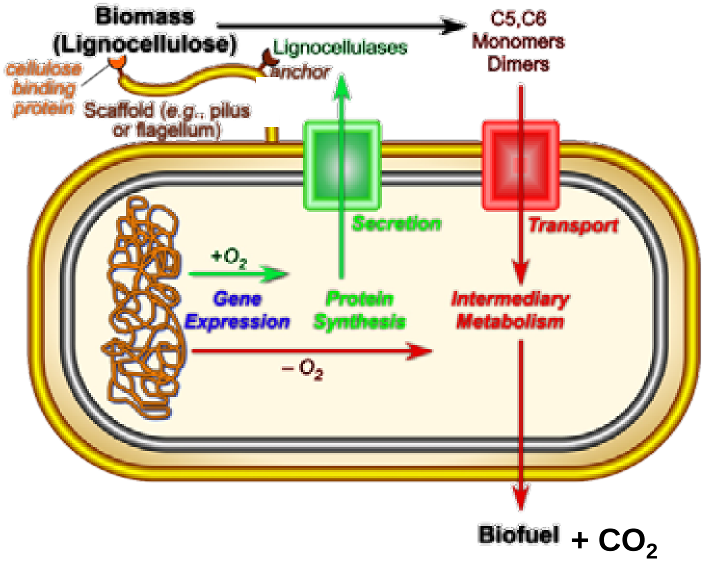


- Tapping genetic variability in wild yeast strains to identify traits that improve sugar (xylose) metabolism, resistance to cellulosic feedstock toxins, or fuel (ethanol) tolerance (*Gasch*)

Improving Microbes for Fuel Production



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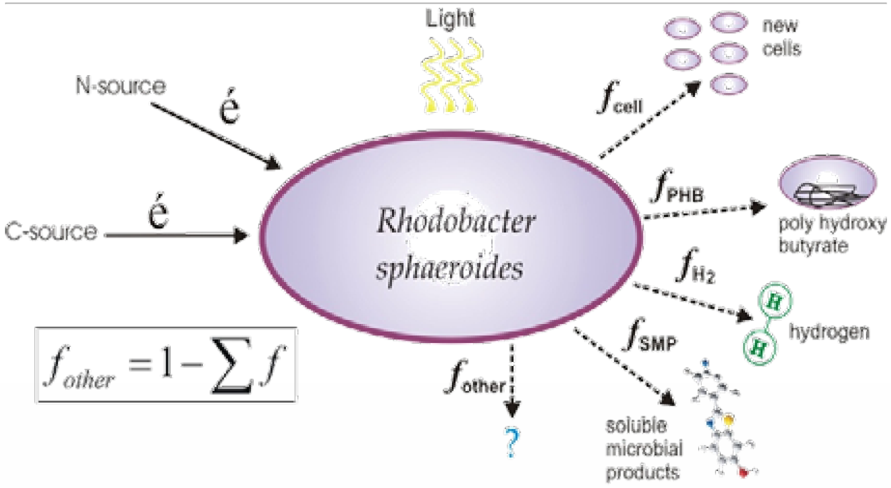
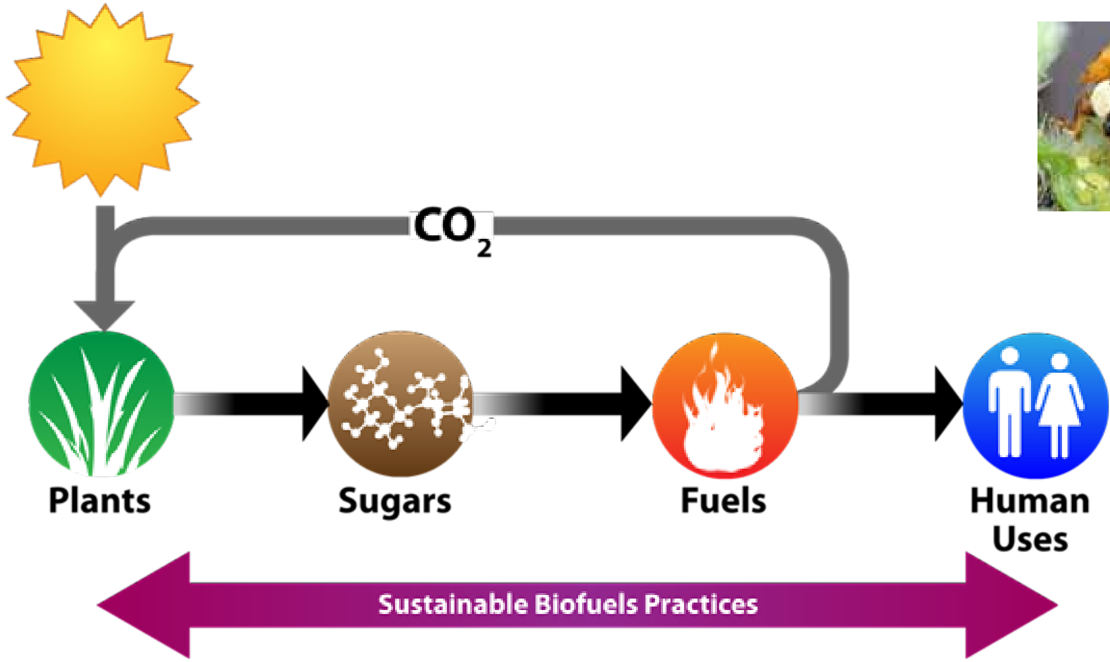
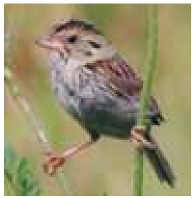
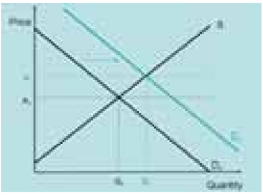


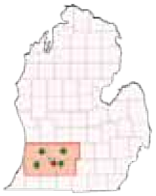
- Monitor and model fate of electrons, CO_2 & cellulosic carbon skeletons to H_2 organic fuels & co-products (*Donohue, Noguera*)

Improving Sustainable Biofuel Production







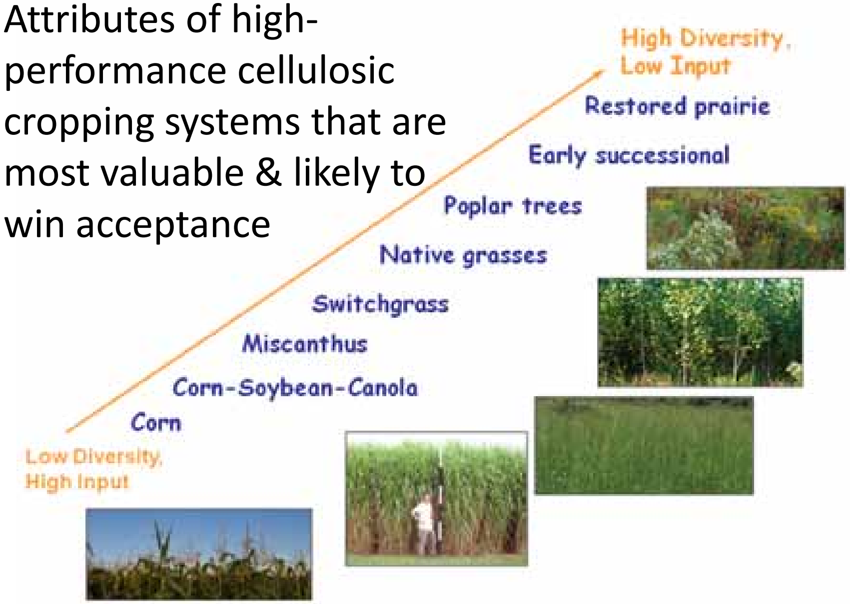


- Obtain knowledge needed to deploy cellulosic biofuel cropping systems that are profitable & environmentally sustainable

Issue	**Approach**
Acceptance	Study output from various production systems
Biogeochemical Responses	Monitor soil, carbon, water & GHG effects
Biodiversity	Quantify pest, disease & ecosystem services
Economic & Environmental	Life cycle scenario testing

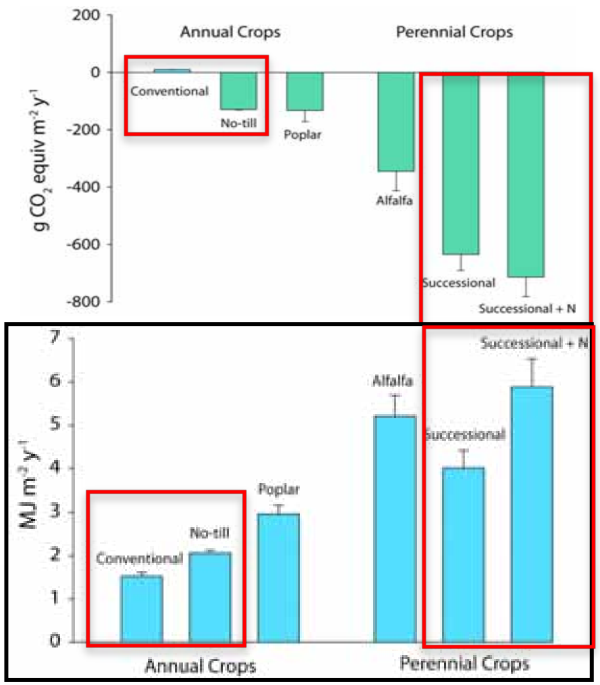
Production & Biogeochemistry of Sustainable Biofuels

Attributes of high-performance cellulosic cropping systems that are most valuable & likely to win acceptance



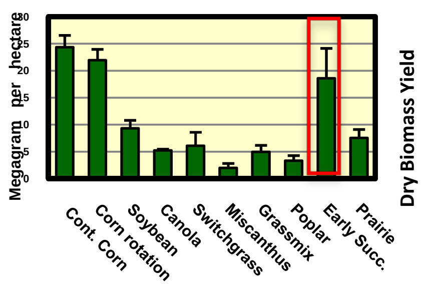
Biogeochemical Responses:

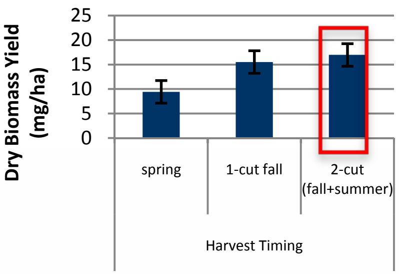
- conventional tillage ~carbon neutral
- successional fertilization benefit offset by greenhouse gases
- early successional least carbon intensive & most land conserving
- energy yields relatively high



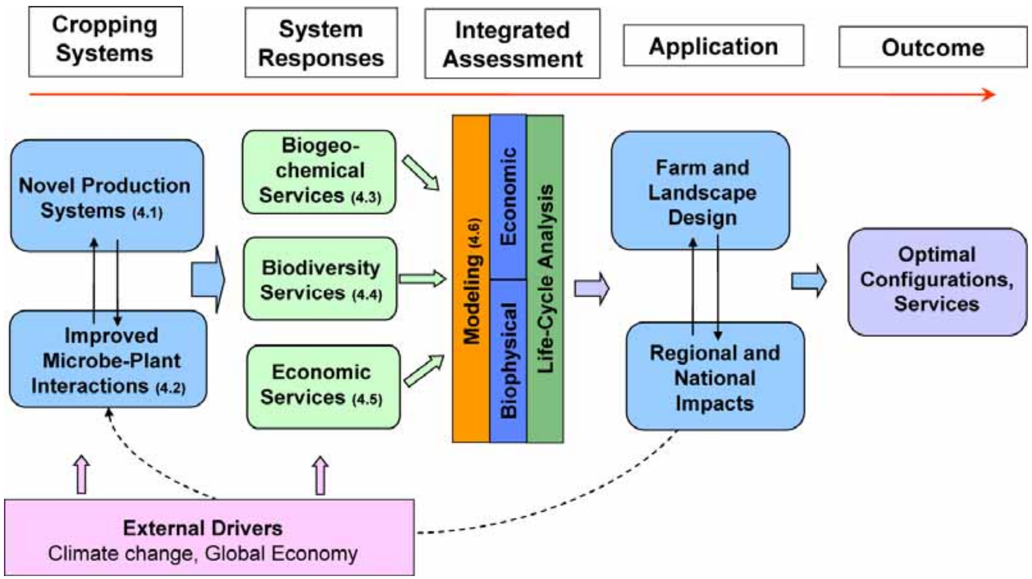
Production Systems:

- Excellent successional performance
- Double-harvest switchgrass yields same as single



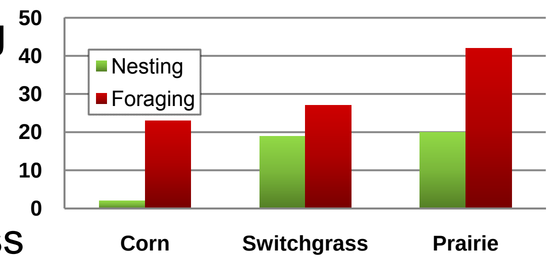


Services & Scenarios of Sustainable Biofuels



Biodiversity Responses: maximize biodiversity delivery (pest & disease control, pollination, wildlife services, etc.)

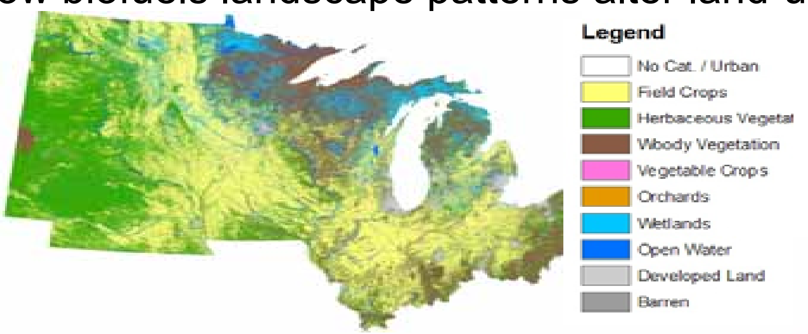
- foraging better in prairie vs. corn or switchgrass



- preliminary conservation concern (4 bird species) in perennials

Principles for multi-scale biofuels modeling:

- Compare landscape & biodiversity
- Agricultural management vs. biodiversity (APEX)
- How biofuels landscape patterns alter land-use



Bringing Our Expertise to the Community

- ~48 biology, chemical, computational or environmental papers
- ~100 presentations (international, national or local) to scientists or other stakeholders (~1 per week since funded)
- ~16 technology disclosures accepted for patent submissions
  - interest in "start up company" based on one technology, licensing conversations for other technologies
  - Advisory board includes leaders from academic, environmental, biofuels (Iogen, POET), automotive (GM), chemical (DuPont), fermentation (Miller) & investing (Piper Jaffray) sectors
- Partners on pending EERE cellulosic pilot/demonstration applications